# Emergence of a virulent wildlife disease: using spatial epidemiology and phylogenetic methods to reconstruct the spread of amphibian viruses

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**Statement of Originality**

I certify that this thesis, and the research to which it refers, are the product of my own work (apart from the collection of data and collation of the Frog Mortality Project database and the collection and analysis of population monitoring data in Picos de Europa as noted in chapters 3 and 5 respectively), and that any idea or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline. I acknowledge the helpful guidance and support of my supervisors, Dr. Trenton Garner and Prof. Richard Nichols.

Signed:

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# Summary of thesis

Ranavirus infection has caused severe disease and mass mortality in UK common frogs for more than twenty years resulting in serious declines in some populations. The pathogen has been studied since 1992. These studies generated two valuable resources exploited in this thesis: an archive of tissues and virus isolates and a database of reports from citizen scientists on ranavirus-consistent mortality. The previous studies yielded modest evidence suggesting that introductions from North America initiated ranavirus emergence in the UK, though little else was known about the pattern of introduction or spread. This thesis conducts a more detailed investigation, extending existing knowledge of ranavirus diversity and spread through molecular epidemiology and phylogenetics, an *in vivo* infection experiment, and *in silico* models.

Non-lethal sampling protocols for ranavirus screening were assessed in a controlled setting and shown to be as effective as traditional protocols. The database of citizen science reports was utilised in spatio-temporal models of the spread of ranavirus disease, finding that ranavirus infection is spreading by transmission between ponds but that new outbreaks are also correlated with both human population density and regional temperatures. The first whole genome sequence from a UK ranavirus is presented. Analysis of the genome shows that it is an isolate of the ranavirus type species, FV3, on the basis of its near identical genome arrangement and a 'supergene' phylogenetic analysis. An unexpected finding was evidence for recent lateral transfer of host DNA into the FV3 genome. A candidate gene survey of European ranaviruses revealed considerable diversity that may explain the variation in virulence and host range in Spain. Two proposed new species of *Ranavirus* are described there - one highly virulent, the other seemingly asymptomatic – and the previously described CMTV is shown to be a likely cause of catastrophic decline across multiple hosts. A lack of monophyly among Spanish ranaviruses and the spatial pattern of incidence suggest recent introduction(s). Together, the evidence presented in this thesis underlines the key role that humans have played in the spread of this group of virulent wildlife pathogens in two European countries.

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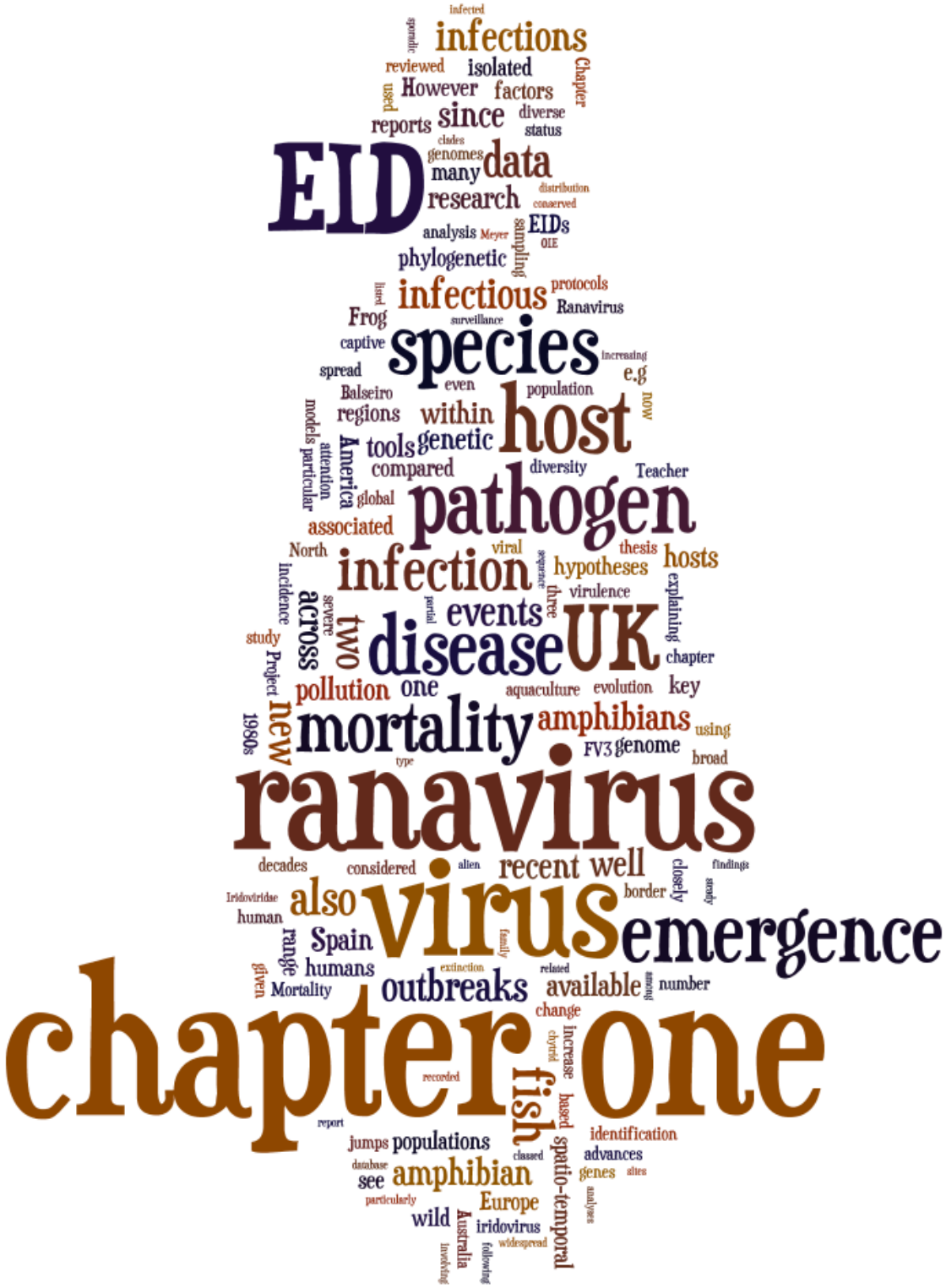
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# 1

# Ranaviruses: emerging pathogens responsible for disease, mass mortality and host declines

Emerging infectious disease (EID) events are proliferating, posing an increasing risk to human health, food security and biodiversity (Daszak et al., 2000). EIDs are therefore the subject of increasing political, medical and research attention (Cunningham et al., 2012). Zoonoses – infectious diseases transmitted from other animal species - pose a particular threat to humans; around two-thirds of human pathogens are zoonotic (Taylor et al., 2001) with RNA viruses of bats particularly noteworthy due to the frequency of

recent host jumps (lyssa viruses, Hendra virus, Nipah virus, severe acute respiratory syndrome (SARS)-like coronaviruses, and Ebola and Marburg viruses) underlining a need to better understand the drivers of spillover events (Wood et al., 2012). EIDs are also a concern for wildlife species (Daszak et al., 2000; Harvell et al., 2002) for whom viral pathogens cause the majority of emergences and anthropogenic factors are key drivers (Dobson and Foufopoulos, 2001). Amphibian conservation status has received particular attention (Alford and Richards, 1999; Stuart et al., 2004; Wake, 1991), not least because EIDs have been proposed as one of six broad hypotheses explaining declines (Collins and Storfer, 2003) along with alien species, over-exploitation, land use change, pollution (usually chemical) and global change.

The plight of amphibians over the last three decades has been severe enough to demand popular media coverage as well as academic interest. Statistics on extinction and status are genuinely alarming. In its most recent assessment, the International Union for Conservation of Nature (IUCN) reported 30% of the 6285 species surveyed as "threatened" (IUCN, 2009). Unfortunately, the true figure is probably nearer 40% given that assessments of a quarter of all species were hampered by deficiencies in available data. Amphibian species have disappeared across the entire taxonomic group and in nearly all regions of the planet (Mendelson et al., 2006) with consequences for humans in terms of ecosystem goods and services (Whiles et al., 2006) such as potential advances in biomedicine and biotechnology (VanCompernolle et al., 2005). Declines and extinctions have been on the increase since the 1980s; they are occurring simultaneously and on a global scale - even in protected areas – earning them special attention (Collins and Storfer, 2003). Such rapid and widespread change results from the interactions between individual factors, but pathogen pollution and the emergence of infectious disease is known to have played a key role (Daszak et al., 1999; Rachowicz et al., 2006).

Pathogen pollution is the introduction of a pathogenic (or potentially pathogenic) parasite to a naïve host species or population (Cunningham et al., 2003). This definition that draws together three of the above hypotheses explaining amphibian declines: infectious disease, pollution and alien species. Defined in this way the term is far-reaching and could include pathogens such as human immunodeficiency virus – introduced to humans from chimpanzees when anthropogenic encroachment increased

interactions between the two species (Sharp et al., 2001). Human-mediated translocations of pathogens or infected hosts represent just a subset of incidents classed as pathogen pollution (see (Cunningham, 1996); precedents include the spread of rabies in the new world (Rupprecht et al., 1995) and rinderpest to Africa (Normile, 2008).

*Batrachochytrium dendrobatidis* (chytrid), the fungal agent of chytridiomycosis has caused extinction and decline across diverse amphibian species in the neotropics and Australia (Berger et al., 1998; Daszak et al., 2003; Lips et al., 2006). Research into impacts of chytrid infection for amphibian hosts began only fifteen years ago but the geographical distribution of a virulent global pandemic lineage (Farrer et al., 2011) now supports the novel pathogen hypothesis as an explanation of rapid emergence (Fisher et al., 2009), which has been likely mediated through human translocations of animals via amphibian trade (Walker et al., 2008). As such, the grave impacts of chytrid infection for amphibian communities in the neotropics is now well documented, but across temperate regions - including Asia, North America and Europe - mass mortality of amphibians due to EIDs is predominantly associated with viral pathogens of the genus, *Ranavirus*, which are less well studied (Duffus, 2009).

## Ranavirus

In 2008 the World Organization for Animal Health (OIE) listed infection with ranaviruses in the Aquatic Code, which requires member states to report outbreaks and follow recommendations designed to limit the spread of listed pathogens through trade (Schloegel et al., 2010). The decision came after approximately two decades of mounting concern among the public, veterinarians, conservationists and aquaculture business owners about the impacts of these pathogens, principally on aquatic vertebrates. Ranavirus is one of five genera of the family *Iridoviridae* - large, icosahedral, nucleo-cytoplasmic viruses with a linear, double-stranded DNA (dsDNA) genome containing many genes compared to most other groups of virus. Although the type species (*Frog virus 3*, FV3) was isolated in 1965 (Granoff et al., 1966) ranaviruses were considered relatively non-pathogenic for the next two decades (Chinchar, 2002). However, in the late 1980s and early 1990s ranaviruses were shown to be key factors in pathology and mortality among fish in Australia (Langdon et al., 1986), and amphibians in the United Kingdom (UK )(Cunningham et al., 1996) and North America (Jancovich et al., 1997). There has been a steady increase in the number and geographical range

that ranavirus publications refer to since then, as well as increases in surveillance effort in some countries, involving networks such as RAVON (http://www.ravon.nl/En/tabid/376/Default.aspx, accessed 13/09/13) of the Netherlands and a long-term citizen science project in the UK (the Frog Mortality Project, FMP).

The known geographic distribution of ranaviruses is now broad; they are found on all continents except for Africa and Antarctica. In the UK and North America ranaviruses have been the subject of ongoing research since the initial causal relationship to mortality events was established in the mid-90s and ranavirus incidence is believed to be widespread (Cunningham, 2001; Green et al., 2002). In contrast, published outbreaks are sporadic in all other regions. A number of reports of ranavirus infection and mortality come from South-East Asia, particularly China, and have mostly been associated with aquaculture facilities. Four of the ten complete ranavirus genomes (*Grouper iridovirus* (GIV), *Tiger frog virus* (TFV), *Rana grylio virus* (RGV) & *Soft-shelled turtle iridovirus* (STIV)) currently available in Genbank were isolated in China or Taiwan and a further one (*Singapore grouper iridovirus* (SGIV)) in Singapore and they are sufficiently genetically differentiated to be considered distinct viral species. Outbreaks have also been associated with aquaculture in South America where infections have been confirmed in several countries (Mazzoni et al., 2009). Australia and mainland Europe have both been home to ranavirus infections in fish since the 1980s (Ahne et al., 1989; Langdon et al., 1986). However infection and disease in Australian amphibians have only been recorded in captive animals. On the other hand, mainland Europe is a hotspot at present for infection, disease and mortality with recent outbreaks in Denmark (Ariel et al., 2009), Spain (Balseiro et al., 2009; Balseiro et al., 2010), Italy (Ariel et al., 2010), the Netherlands (Kik et al., 2011), Belgium (Sharifian-Fard et al., 2011) and Germany (Stöhr et al., 2013); yet prior to 2007 there had been just one confirmed case in Croatia (Fijan et al., 1991). The current picture of sporadic outbreaks in many regions may be a consequence of actual incidence, inadequate surveillance or a lack of publicly available data in the past but the OIE requirement that states should file annual reports of cases means that better data will soon be available.

It is important for the understanding of the emergence of ranavirus infections to note that ranaviruses have an extraordinarily broad host range encompassing amphibians,

reptiles and fish. Amphibians have been the target of most research effort given the severity of disease and mortality that this group can sometimes experience and the incidence across diverse amphibian families (see figure 1.1). However there is considerable variation in susceptibility to infection between life history stages, within populations, and across the phylogenetic tree (reviewed by Miller et al., 2011). In reptiles, ranavirus infection and disease have now also been described chelonians (turtles and tortoises) from many parts of the world and there have been occasional cases in snakes and lizards (reviewed in Marschang (2011) and Alves de Matos et al., 2011). Fish are the primary hosts of GIV-like ranaviruses but can also be infected by amphibian-like ranaviruses (ALRVs): for example, *Epizootic hematopoietic necrosis virus* (EHNV) is classed as an ALRV (Jancovich et al., 2010) even though it is only thought to infect fish in the wild, whilst ranaviruses from a frog tadpole and a stickleback (both wild) were considered identical (Mao et al., 1999), and fish are sometimes susceptible to amphibian ranaviruses when experimentally exposed (e.g. See Moody & Owens (1994) in contrast to Jancovich et al. (2001)). Finally, the fact that members of the *Lymphocystivirus*, *Megalocytivirus*, as well as GIV-like ranaviruses, parasitise fish hosts, points to fish as the ancestral vertebrate host of ranaviruses and a number of alternative hypotheses involving host jumps from fish to amphibians have been discussed (Jancovich et al., 2010).

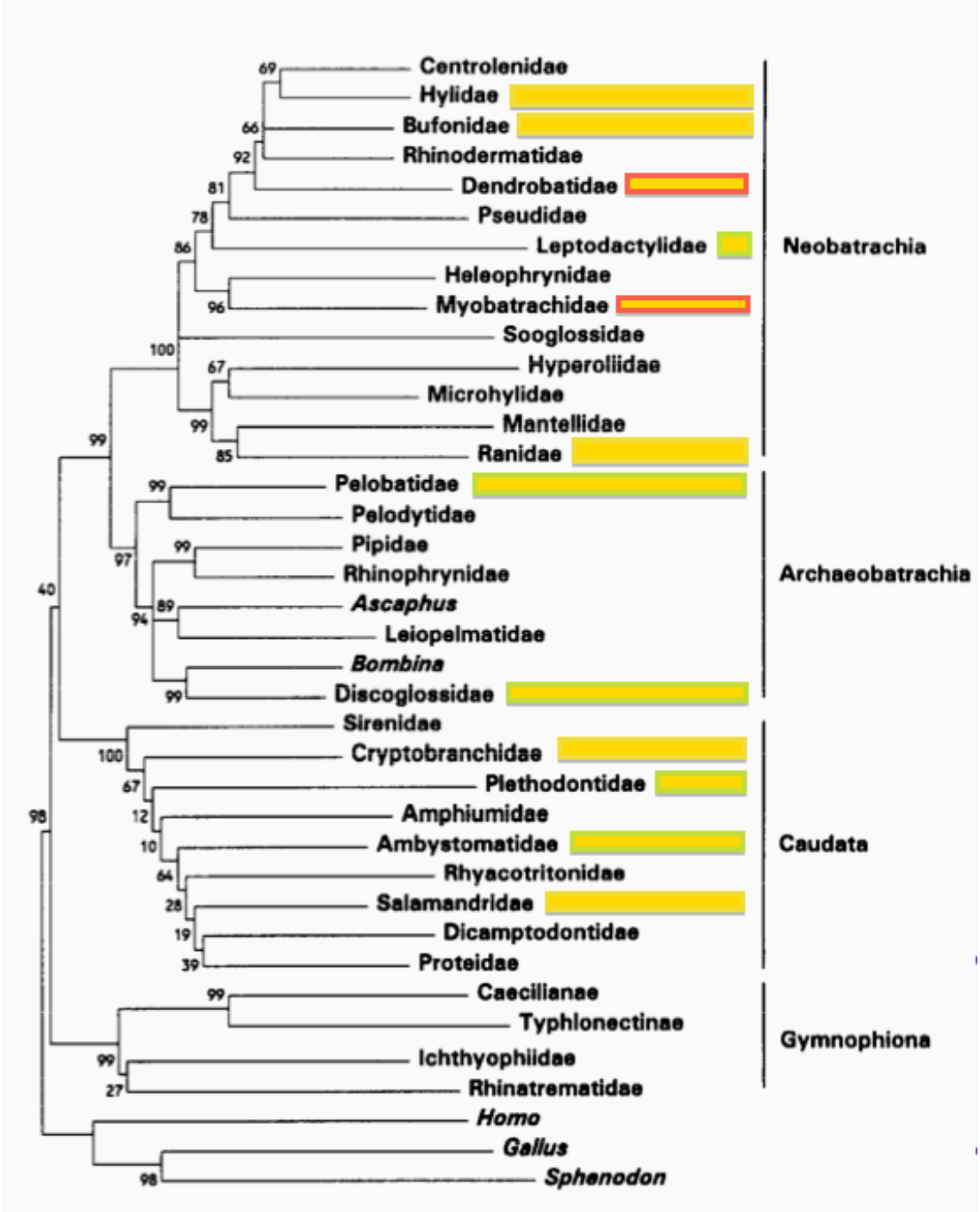



Figure 1.1. Confirmed ranavirus infections in families of the Amphibia. Green border = infections in wild populations only, red border = captive populations only, and yellow border = both wild and captive infections. Phylogeny is adapted from Hay et al. (1995) with reference to Miller et al. (2011).

## Tools used to study pathogen emergence

The power of phylogenetic trees as tools in the reconstruction of the spread of infectious diseases has long since been recognised (e.g. see Holmes et al., 1995). Genetic patterns within pathogens can reveal geographic sub-structuring – that some populations are more closely related than others; the genetic diversity within clades can support inferences about jumps to new host species, and identification of genetic variants (e.g. specific clades, haplotypes or alleles) associated with virulence or tissue tropisms can improve mitigation or diagnostic tests (Holmes, 1998). The combination of evolutionary biology, immunology and epidemiology driving infectious disease behaviour has been named pathogen 'phylodynamics' (Grenfell et al., 2004) and its study has required the emergence of a new discipline combining the analysis of genetic diversity and pathogen ecology alongside the exploitation of new analytical tools and increased computational power (reviewed by Pybus & Rambaut, 2009).

Phylogenetic studies of ranaviruses have utilised candidate genes with some success in order to reconstruct ranavirus relationships (Hyatt et al., 2000) allowing rapid categorisation or differentiation of ranaviruses following outbreaks (Marsh et al., 2002), identification of novel genotypes (Balseiro et al., 2009) and identification of geographically variable selection (Ridenhour and Storfer, 2008). A steady increase in availability of ranavirus genomic data has started to expand the scope of ranavirus study not only through more robust phylogenetics (e.g. see Jancovich et al., 2010; Eaton et al., 2010; Mavian et al., 2012) but also in terms of understanding genome arrangement (Eaton et al., 2010) and evolution (Jancovich et al., 2010) as well as the functional biology of virulence (Chen et al., 2011; Rothenburg et al., 2011). Recent and ongoing technological advances of next generation sequencing (NGS) methodologies and the relatively small size of ranavirus genomes (105kb to 140kb) mean that sequencing many ranavirus genomes simultaneously is now easier and more affordable. These new datasets will alter the way ranavirus research is conducted, with possibilities for studying genome evolution, unraveling gene function and explaining virulence, and even undertaking experimental evolution.

These advances can be integrated with spatial epidemiology, another field where exciting developments are boosting researchers' toolkits. Statistical tools for the analysis of point processes as models to explain infectious disease data are proliferating.

Many of these tools are freely available for implementation within the statistical language R. An example is the suite of functions within the package Surveillance (Meyer et al., 2012), which provide new tools in the underdeveloped area of spatio-temporal analysis. Included are options for modeling the risk of a disease outbreak arising at a given spatio-temporal point in continuous space (Meyer et al., 2012). Patterns in genetic data can be sought to assess and validate predictions from spatio-temporal models (Garner et al., 2012).

This thesis utilises these new approaches to build on existing knowledge of ranaviruses in Europe and to answer questions about emergence in the UK and Spain in particular. The UK was one of the first sites where severe mortality events with ranavirus aetiology were investigated, using molecular screening protocols and cell culture techniques to provide evidence of infection (Cunningham et al., 1996). A valuable resource is the collection of archived samples from diseased animals and cultured viruses maintained since 1992 (Cunningham et al., 2007a; Cunningham et al., 1996). A second key resource for this thesis is the database initiated by the UK Frog Mortality Project, which was also established in 1992 to collate reports of events where ranavirus pathogenicity appeared to have caused significant mortality.

Previous genetic analysis of UK ranavirus isolates has provided some important insights. A comparison of amphibian and fish ranaviruses based on morphological characteristics, restriction fragment length polymorphisms (RFLPs) and a partial 586bp sequence of the 3' end of the conserved Major Capsid Protein as well as 100bp downstream, revealed two UK viruses isolated from common frogs were very closely related to FV3 from the USA, suggesting a trans-Atlantic introduction of virus into the UK (Hyatt et al., 2000). This insight into the infection has been extended by recent major projects to investigate host range and infection dynamics (Cunningham et al. 2007a; Cunningham et al., 2007b; Duffus, 2009) and host population responses to recurrent infections (Teacher et al., 2010; Teacher et al., 2009a; Teacher et al., 2009b). This thesis focuses more on viruses than the hosts or pathology. It also makes comparisons between the UK and Spain, where emergence of outbreaks appears to be more recent, and to have occurred in a national park rather than in domestic gardens.

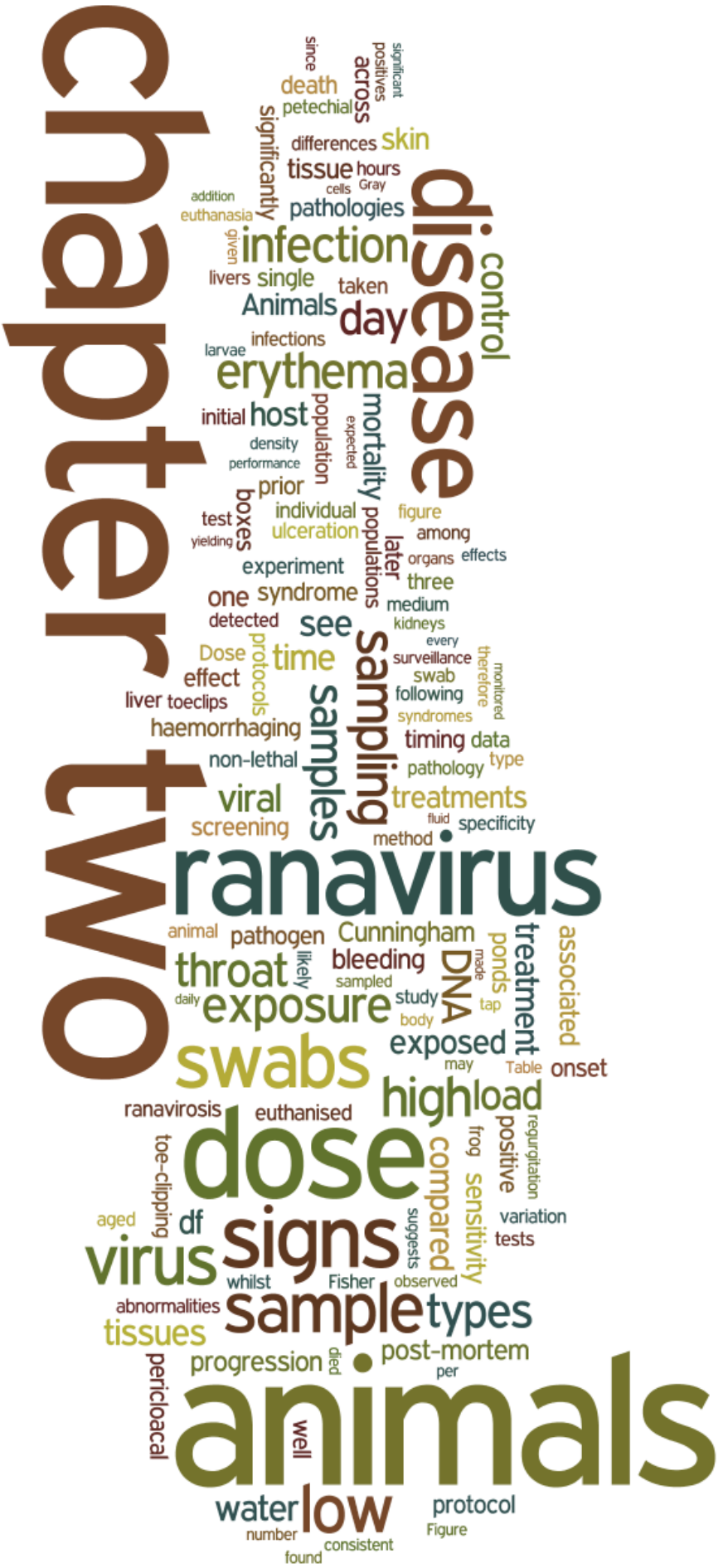
Chapter 2 describes an infection experiment designed to assess alternative sampling protocols for ranaviruses and the progression of disease. Common frogs were exposed to a UK ranavirus isolate from 1995 (RUK13) in three treatments (mock, low dose and high dose). Animals were closely monitored for possible signs of disease and timings recorded. Swabs, toeclips and visceral organs were all sampled and used in quantitative PCR screens for ranavirus to compare lethal, non-lethal and invasive sampling protocols.

Chapter 3 implements findings from chapter 2 in order to filter data from the Frog Mortality Project database for ranavirus-consistent reports of amphibian mortality based on the most reliable indicators of ranavirosis. Alternative hypotheses for the UK emergence of ranavirus are compared using two-component spatio-temporal models.

I report the first whole genome sequence data from a UK ranavirus in chapter 4. Results of *de novo* assembly of 454 reads from a virus isolated in 1995 as part of the initial investigation of ranavirus-related mortality events are presented and compared to the type ranavirus species, FV3. Phylogenetic analyses based on a set of 26 genes conserved across the family *Iridoviridae* as well as a single candidate gene are used to determine the relationship between UK and other ranaviruses.

Chapter 5 also uses virus genetics - this time partial coding sequences from two loci - to examine ranavirus diversity in Spain following recent disease outbreaks and mortality events. I extended existing ranavirus sampling in northern Spain, collecting samples from multiple, diverse hosts and sites in two regions and constructed phylogenetic trees using two loci and their concatenated alignment. Findings from phylogenetic analyses were compared to ecological factors such as host range, mortality and host population status.

Finally in chapter 6, I discuss the overall context of this work, drawing together findings from individual chapters to make more general comments on ranavirus emergence and prospects for future research.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# 2

# Experimental assessment of sampling strategies and disease progression following ranavirus infection

## Abstract

Improved characterization of the disease process associated with ranavirus infection would enable *a priori* filtering of ranavirus-consistent reports and provision of better guidance to citizen-scientists about when, where and how to carry out surveillance. In addition, detection of ranaviruses is an ongoing challenge to

mitigation and research because existing sampling protocols restrict the collection of fundamental data. This study aims to address both issues by examining the ecology of ranavirus infection in a controlled setting and comparing sampling protocols (traditional, non-lethal and non-invasive).

Juvenile common frogs were bath exposed to either high ($10^{4.6}$ $TCID_{50}$/ml) or low ($10^{3.3}$ $TCID_{50}$/ml) doses of a ranavirus isolate, RUK13, or virus culture medium only, and monitored daily for signs of disease. Swabs (throat and pericloacal) were taken eight days post-infection. Toe, liver and kidney samples were collected post-mortem and all samples were quantified using real-time PCR.

Signs of pathology consistent with ranavirus infection were identified. Erythema of the lips, regurgitation, bleeding and muscle abnormalities were only noted in animals from exposed treatments and were highly significantly associated with ranavirus exposure. Both non-lethal and non-invasive sampling protocols were shown to be effective in screening for infection in diseased animals: toeclips and pericloacal swabs performed well in terms of specificity and sensitivity when compared to sampling livers, which are considered a gold standard. Dose impacted significantly on the timing of signs of disease: lip erythema (1.4 days, $p=0.02$), other erythema (3.2 days, $p=0.01$), petechial haemorrhaging in the skin (3.6 days, $p<0.0001$) and regurgitation (2.9 days, $p=0.02$) occurred significantly later in low dose animals than high dose. Ulceration was a late-onset sign in both ranavirus treatments and suggests ulcerative and haemorrhagic syndromes may be part of a disease continuum.

Viral load increased through time until death: pericloacal swabs returned significantly higher virus titres in high dose animals than low dose (ANOVA; $F=12.35$, $df=1,36$, $p<0.005$) but post-mortem viral load did not vary significantly between treatments across any of the three tissues sampled. Higher concentration inoculates yielding earlier onset disease and higher viral loads amongst diseased animals could explain more severe declines in large populations experiencing recurrent disease.

# Introduction

Ranaviruses are large dsDNA viruses of the family *Iridoviridae,* which are distributed globally. They have a broad host range (reviewed in Miller et al., 2011) -infecting fish, reptiles, and amphibians – and often cause severe disease and mass mortality. Amphibian mortality associated with ranavirus infection is usually most

severe in larvae and just after metamorphosis, but can also occur in adults. Infection can drive long term population declines (Teacher et al., 2010). Ranavirus infection is widespread in England where mortality and disease is almost exclusively reported in adult common frogs (*Rana temporaria*) (Cunningham, 2001). Host response to ranavirus infection in the UK has generally been measured in terms of carcass counts (see 'The Frog Mortality Project (FMP): surveillance by the public' below) or host population dynamics. Both outputs are extremely variable. Some frog populations experience recurrent die-offs, whilst at other ponds there may be just a single mortality event, which can have contrasting outcomes: either extirpation of the population or no apparent effect (Teacher et al., 2010). A better understanding of the disease process at an individual level may help explain variability in population level responses.

## Ranavirosis

A member of the *Iridoviridae* was established as the cause of many of the unusual frog mortality events in the UK following a thorough microbiology and pathology investigation (Cunningham et al., 1996). Patterns of pathology corresponding to two disease syndromes were described: ulcerative syndrome typified by skin ulceration - especially of the femoral skin - and necrosis of the digits and limbs, and haemorrhagic syndrome typified by systemic haemorrhaging - especially of the skeletal muscle, gastrointestinal tract and reproductive organs. Skin erythema and sloughing, poor body condition and emaciation, and lethargy were found with both syndromes, whilst congestion of the lungs, liver and kidneys was noted occasionally.

In addition to the haemorrhaging and organ congestion, infected larval and metamorphosing amphibians are known to swim erratically, exhibit lethargy and a lack of equilibrium, and develop erythema and swelling of the body and legs (Gray et al., 2009). Empty guts and enlarged gall bladders 'consistent with anorexia' are also found (Gray et al., 2009).

The apparently systemic nature of some infections suggests that virions disperse widely throughout the body. Cunningham et al. (2008) used immunohistochemistry to examine ranavirus tissue tropisms in infected hosts. Epithelial cells, fibrocytes, lymphocytes, sinusoidal lining cells of the liver and melano-macrophages all frequently contained virus; one difference between 'ulcerative syndrome' and 'haemorrhagic

syndrome' is that a greater number of tissue-types contained virus in the latter. Disease syndrome should therefore be expected to influence detection probabilities among tissues that are sampled for ranavirus. At the same time, the lack of specificity of some signs means diagnosis of ranavirosis is not possible based only on clinical signs (Chinchar and Mao, 2000) and diagnostic tests are required. Microscopy (TEM), serology (ELISA), PCR, Restriction Fragment Length Polymorphism and protein profiles have all been utilised in ranavirus diagnostics and strain identification (Chinchar and Mao, 2000).

## Diagnostics

The choice of tissue sampled then will affect the likelihood of detecting infection. In addition, the use of visceral organs necessitates either opportunistic sampling of carcasses or removal of animals from the wild, both of which restrict the frequency of opportunity to estimate even basic epidemiological quantities such as prevalence. This has been the case for ranavirus sampling which has largely relied on PCR of part of the Major Capsid Protein (MCP) gene using DNA extracted from visceral organs (usually livers or kidneys).

Recently, toe clipping has been shown to be an effective alternative to visceral organs in wild-caught anurans (*Rana clamitans*) from several populations in Ontario, Canada (St-Amour and Lesbarreres, 2007), offering a less restricted and non-lethal sampling protocol for ranavirus screening. Tail clipping of caudates and larvae is a similar method that has been used to sample Ambystomatid salamanders in populations infected with ATV (Brunner et al., 2004), though experimental evidence suggests that this method may underestimate true ranavirus infection prevalence in wild populations (Greer and Collins, 2007). A controlled assessment of tail-clips and swabs from larval anurans also showed that whilst non-lethal sampling protocols may be useful they are likely to underestimate true prevalence (Gray et al., 2012)

An additional concern about toe clipping is animal welfare. The method is a widespread and longstanding tool used by ecologists to mark amphibians in studies of disperal and life history, but concerns over the ethics of this technique, and its impact on individual survival, have been expressed recently (Parris and McCarthy, 2001; McCarthy and Parris, 2004; May, 2004). In assessing the ethics of using toe-clipping for

disease-screening, it is arguably more valid to compare it with the options available for collecting DNA for population genetics studies, since both objectives require sample collection whilst toe-clipping for mark-recapture is a means of identifying animals. To this end, one can make use of the reasoning of Parris et al. (2010), who used a formal decision-making technique to assess non-lethal sampling protocols for population genetics studies. Larval tail tipping, removal of whole animals (larvae), adult toe clipping, and buccal swabbing of adults were compared as strategies to obtain amphibian DNA from multiple species (including *Rana temporaria*). Buccal swabbing was ranked higher than toe clipping across every indicator used, though it did cause local bleeding in some animals. Swabbing is a generally less controversial, non-invasive sampling technique that is already used routinely in screening amphibians for *Batrachochytrium dendrobatidis* infection and has also been shown to be useful in screening anuran larvae for ranavirus (Gray et al., 2012).

Although the performance of swabbing as a ranavirus sampling protocol has not yet been properly assessed in adult anurans, virus is present throughout multiple tissues of animals with haemorrhagic syndrome and animals with ulcerative syndrome suffer striking skin lesions. It is reasonable to assume that the mouth, nostrils and cloacal vent and skin might be good targets to sample virion shedding and that the volume of shed virions might vary among animals according to their signs of disease.

## Dose-response

The dose of an infectious agent in an initial inoculate is another variable expected to influence pathogen detectability as well as disease progression in a host. Higher initial doses could either reduce the time available to mount an immune response prior to the onset of clinical disease (and capacity of immune defenses to mediate clearance once they are operational) or reduce the amount of time a host has to ameliorate pathogen damage. The concentration of the initial inoculate may therefore determine whether a host develops an acute disease syndrome or a more chronic presentation. In cases where there is a critical threshold in host tolerance, higher doses should reduce the time required to reach the threshold and are therefore likely to reduce survival times and time to onset of signs (e.g. see Timms et al., 2001).

If hosts are exposed to low initial doses of a pathogen, which replicates slowly, then a diagnostic test will need to be very sensitive to avoid false negatives. Desirable properties of screening tests can include the ability to a) carry out surveillance to proactively scan for pathogen emergence rather than just post-hoc diagnosis after an outbreak, b) screen for asymptomatic infections, c) pinpoint infection and enable clinical control, and d) evaluate the effectiveness of interventions (Banoo et al., 2010). A test's performance and operational characteristics should ideally be optimized. The key performance attributes are sensitivity (how likely an infected individual is to be identified as such by the test) and specificity (how likely an uninfected individual is to test negative), whilst operational characteristics include ease of use.

There is experimental evidence that dose is an important factor explaining ranavirus virulence in salamander larvae (Brunner et al., 2005). The relationship between dose and virulence is not straightforward since increasing host density can negate the dose effect. This suggests a trade-off between the costs of infection and increasing host density whereby initiation of an immune response mitigates density induced stress (Echaubard et al., 2010). Dose-response studies of ranaviruses in amphibians have focused on larvae - probably due to the more straightforward housing and husbandry of animals - but effects are unknown in adults where different responses might be expected given their more sophisticated immune system (Maniero et al., 2006).

## The Frog Mortality Project (FMP): surveillance by the public

The FMP is a collaboration between scientists and members of the public, administered latterly by an amphibian conservation organization (Froglife), which has yielded more than 5000 reports of amphibian morbidity and mortality. An obvious strength of the FMP is its size: its database comprises more than 5000 reports containing information in up to 169 fields. Acquiring such a huge volume of records was made possible through participation of a large number of citizens. Data cover site information, the mortality event, the pond and its setting, and more. However, the main focus of the project is disease; to obtain key data, the citizen-participants (often the pond owners) are asked to confirm the presence or absence of certain lesions or signs, many of which are thought to characterise ranavirus infection. The reporting system was essentially report-led: the contents of early, unsolicited reports and post-mortem examinations of carcasses submitted to ZSL guided the content of the

disease section of the reporting form, which has altered little over two subsequent decades.

One objective of the current study is to obtain clearer evidence in order to provide better guidance to the citizen-scientists about when, where and how to carry out surveillance for ranavirus infection - maximising the information gained from their effort and concern. A second objective is to assess lethal, non-lethal and non-invasive sampling protocols in the context of two variables that are expected to influence ranavirus detectability: dose and disease progression. Swabbing sites on the amphibian body from where they are likely to be shedding virions into the environment is expected to be an effective method of screening for ranavirus. Additionally, if pathogen burden is proportional to severity of disease then inoculate dose and time since exposure will also affect the sensitivity of sampling protocols.

# Methods

## Source and rearing of animals

All animals used in this study were juvenile (1.5 year old) common frogs (*R. temporaria*). Animals were reared from the egg stage in a dedicated outside area at the Zoological Society of London (ZSL). Two part-clutches were collected in March 2010 from an artificial pond in SE England with no known history of ranavirus infection. The egg masses were held in 84L clear plastic boxes (dimensions; external: 710 x 440 x 380; internal: 605 x 370 x 355; length x width x depth in mm) containing approximately 35L of tap water. Tap water was aged for 24 hours to de-chlorinate it. A small amount of oxygenating plant material from the source pond was also added to the boxes.

Water was changed twice weekly (85% of water replaced) following tadpole hatching and throughout early development when tadpoles were non-feeding (prior to Gosner stage 23, approximately three to four weeks post-hatching). From Gosner stage 23, spare tubs of aged water were maintained and the tadpoles were moved over to a new tub every two days by gentle netting. Animals were fed ad libitum using Tetra tabimin pellets from approximately Gosner stager 23.

House bricks were provided as platforms for animals to aid egress from water during metamorphosis. Animals were transferred to a biphasic (terrestrial and aquatic) enclosure once legs were fully developed and their tails had receded to one third of full length.

Biphasic enclosures were set up in 84L plastic boxes with coconut soil as a substrate. Two 0.3L boxes were buried in the substrate and filled with aged tap water and a stone. Small upturned plastic plant pots (5cm in diameter) with cut outs allowing entry were provided as cover objects. Animals were housed at a density of 100 animals per box and fed hatchling brown crickets ad libitum on alternate days. Water was topped up and refreshed regularly. Animals were overwintered from October 2010 to February 2011 at a stable temperature of 4°C in the same 84L boxes containing a layer of substrate and filled to the top with fallen leaves autoclaved to minimise fungus growth. Following hibernation, animals were housed as previously and at the reduced stocking density of 50 animals per box until the experiment start date in September.

## Virus production and quantification

The virus isolate used in this study was RUK13, isolated from a dead and diseased common frog in Suffolk, England, in 1995 and previously shown to cause infection and disease experimentally (Cunningham et al., 2007a). Following isolation (described in Cunningham et al., 2001) the virus was passaged three times on confluent fathead minnow (FHM) cells in maintenance medium (EMEM plus 10% FBS plus 1% L-glutamine) and spun at 800g with the pellet discarded to remove cell debris.

Virus titre was calculated using a $TCID_{50}$ (50% Tissue Culture Infectious Dose) protocol. Fully confluent layers of FHM cells were prepared in a 96-well flat-bottomed tissue culture plate and 200µl of maintenance medium. Virus (having undergone the same number of freeze-thaw cycles and storage as virus used for exposures) was serially diluted in the same medium with dilutions from $10^{-3}$ to $10^{-10}$ used to inoculate cells. Wells were inoculated with 100µl of diluted virus in replicates of ten for each dilution with each dilution on a different row of the plate. The remaining two wells on each row were inoculated with 100µl of maintenance media only as a control. Wells were monitored daily for cytopathic effect (CPE; plaques

forming in the cell layer) and after five days each well was scored either positive of negative for CPE. $TCID_{50}$ for the virus stock was then calculated using the Reed and Muench method (Reed and Muench, 1938) giving a stock virus solution containing $10^{5.6}$ $TCID_{50}$/ml.

## Acclimation, Housing and husbandry

Animals were acclimatised in their original enclosures for two days following transfer to the designated room. They were then allocated randomly to treatments, weighed, and transferred to individual boxes prior to two further days of acclimation. Ninety animals (30 per treatment) were individually housed in 1.6L boxes (internal dimensions = 150 x 110 x 95 (length x width x depth in mm)) with a non-airtight lid for the duration of the experiment. The box was lined with a sheet of single-ply tissue paper folded and saturated with aged tap water and contained a single, upturned, 50mm plastic plant pot with cut-out entrance for cover. Boxes were individually numbered and arranged in a single layer (15 per shelf).

The room was climate controlled with lighting set to a constant 12-hour day/night cycle. Environmental conditions (temperature and humidity) were monitored daily for two weeks prior to and throughout the experiment. Mean temperature in the room was 20.2°C (min=18.6°C, max=21.1°C) and mean humidity was 59% (min=47%, max=79%). An additional box – set up exactly as above but housing a thermometer and no frog - was used to monitor proximate conditions (mean temperature=19.9°C, min=18.8°C, max=20.5°C).

Boxes were rotated daily (one position forwards and one to the right; boxes at front of shelf to back, boxes at right hand edge to leftmost column) to ensure there were no persistent effects of location on frog responses. Shelves were also rotated (top to bottom and left to right) on racking on alternate days. Animals were fed 10 first instar brown crickets every second day. Gloves were changed between treatments. Boxes were cleaned and animals weighed on every fourth day beginning on the day of exposure.

## Exposure

All experimental procedures, including euthanasia, were done under Home Office license (PIL# 80/12138; PPL# 80/2214) and after full ethical review by ZSL's Animal

Ethics Committee. Animals were randomly assigned to three different treatments: control, low dose, and high dose. Two 1L sterile bottles of exposure medium were prepared for each treatment. The control exposure medium consisted of cell culture maintenance media diluted in aged tap water. High dose exposure medium consisted of stock virus solution diluted in aged tap water. The low dose exposure medium involved two dilution steps: stock virus was diluted in cell culture maintenance medium, followed by a second dilution step with aged tap water. This procedure ensured that animals from all treatments were exposed to identical concentrations of cell culture maintenance medium. The final virus titre was $10^{4.6}$ $TCID_{50}$/ml of virus for the high dose treatment and $10^{3.3}$ $TCID_{50}$/ml for the low dose treatment.

Animals were bath exposed in individual containers (0.07L Really Useful Boxes). 65ml of the appropriate exposure media was pipetted through holes in the container lids and animals were exposed for 7 hours, after which time the containers were drained and animals rehoused.

## Sampling

Animals were monitored daily and scored for changes in spontaneous behaviour, behaviour during handling (when applicable), food intake, stools, and body condition. Animals were also monitored for signs of disease typical of ranavirus infection (see Introduction) - skin erythema, petechial haemorrhaging, ulceration, bleeding consistent with internal haemorrhaging, emaciation, lethargy - as well as any other physical changes.

Swab samples from the throat and pericloacal regions of each animal were collected at day 8 (and at day 16 from one surviving animal) using sterile dry swabs (MWE). Throat swabs were rolled across the skin of the throat to cover the skin surface entirely as well as the lips and nostrils. Pericloacal swabs were obtained by rolling the swab tip forwards and backwards across the cloacal entrance three times. These two swabbing protocols were chosen as they are assumed to be focal sites for viral shedding and since they enable straightforward and repeatable sampling.

Day 8 after exposure was predicted to be the approximate midpoint of the experiment based on previous infections in adult common frogs (Cunningham et al., 2007). Half

of the animals in the control and low dose treatments were selected randomly for euthanasia (lethal overdose of MS222 following UFAW guidelines). Five of the animals in the low dose treatment died overnight prior to euthanasia so eight (of 26) animals were sacrificed along with 11 (of 22) control animals.

Dead animals were examined within twelve hours of death for skin lesions and abnormalities, and post-mortem throat and pericloacal swabs taken. The carcasses (frozen or within three days of death) were then subjected to a standard post-mortem examination (see Appendix A), which included careful scrutiny by eye and under a dissection microscope. Whole organs or tissue biopsies were taken from a range of tissues including liver, kidneys, and 3rd toe of right hind-foot and samples were frozen at −20°C.

## Estimation of viral load

Samples were defrosted and small pieces (20mg) of the kidneys, livers and toes prepared. Control tissues were prepared alongside samples. Positive control tissues came from carcasses submitted to ZSL and the subject of four consecutive positive ranavirus screens (conventional PCR using primers from Mao et al., 1996). Negative control tissues came from two year old animals reared at ZSL from spawn without signs of infection or ill-health and the subject of four consecutive negative ranavirus screens using the same method as for positive controls.

All tissues were disrupted by beating with 5mm beads at 15Hz for 20s in a Qiagen Tissue Lyser II with the digestion mixture specified by the Promega Wizard SV 96 Genomic DNA Purification System protocol for animal tissues. They were then digested overnight for 18 hours at 56°C before proceeding with the remainder of the protocol. DNA was eluted in a single step leading to a total volume of approximately 250µl. The tips of swabs were cut off using a new scalpel blade for each sample and placed in individual microcentrifuge tubes with the Promega Wizard digestion mix (as above) plus 0.03-0.04g of 0.5mm silica microbeads. Negative extraction-controls were generated by immersing swabs in sterile water; positive control swabs were immersed in cultured RUK13. Control swabs were then subjected to a freeze-thaw cycle similar to experimental swabs. All swabs were beaten for 45s at 30Hz in the tissue lyser and incubated overnight for 18 hours at 56°C before proceeding with the

Promega Wizard SV 96 Genomic DNA Purification System protocol for animal tissues as above and single elution of 250µl. DNA concentration of each sample was measured using 1µl aliquots on a NanoDrop 2000 microvolume spectrophotometer.

A quantitative polymerase chain reaction was used to estimate viral load. I used DNA primers designed by Holopainen et al (2011) to amplify a 93bp fragment of the DNA polymerase largest subunit. SYTO13 was used as the dsDNA reporter dye to enable real time quantification given its improved performance over SYBR green in terms of reaction inhibition at high concentrations (Monis et al., 2005). Each sample was screened in duplicate wells using a reaction mix of 12.5µL of Promega GoTaq Hot Start Colourless Mastermix, 0.625µL of 10µM forward primer, 0.625µL of 10µM reverse primer, 0.25µL SYTO13 (500µM stock), 0.5µL ROX, 5.5µL of nuclease free water and 5µL of template DNA.

Samples were run on 96 well plates with negative controls (duplicate wells with nuclease free water as template) and standards. Standards were generated from cultured stocks of another ranavirus isolate - RUK11 (Cunningham et al., 2007a) - quantified following the same protocol described above for RUK13, prior to DNA extraction using a DNAeasy Blood and Tissue Kit. A 300µl aliquot of cultured RUK11 was placed in a clean microcentrifuge tube before adding 20µl of proteinaseK and proceeding with the Purification of Total DNA from Animal Blood or Cells (Spin-Column) protocol from steps 2 to 7 with a single elution step of 200µl. Standards were added to each plate in a dilution series in duplicates. Plates were run on an Applied Biosystems Stepone Plus thermocycler using the same settings as Holopainen et al. (2011). If one of the replicates failed to amplify or there were large standard deviations of mean CT values they were rerun in duplicate to verify the result.

Inhibitors of PCR reactions are both diverse and abundant and can result in inaccurate quantification or false negatives (Wilson, 1997). The presence and extent of inhibition of PCR reactions was not quantified nor controlled for in this study as it was not considered a confounding effect for the comparisons and analyses undertaken. Dose effects were compared by tissue and - although inhibition may vary across tissue types - for a given tissue type, inhibition is assumed to be consistent between samples, aided by approximate standardisation of tissue weight. Stool and faecal samples can vary

significantly in component levels between individual samples due to factors such as nutrition, gut microbiome, lifestyle and environment (Schrader et al., 2012). Some of these factors could reasonably also affect samples of visceral organs and swabs but are considered negligible here given the consistency of environment, diet and general husbandry experienced by all animals right from the egg stage (see 'Source and rearing of animals', page 31). Comparisons between tissue types may be seriously confounded by PCR inhibition and therefore complicate attempts to study tissue tropisms. However, inhibition does not confound the comparison of alternative sampling protocols undertaken here. Although PCR inhibition could cause variation in the sensitivity and specificity of screening between sample types this would represent an explanation of the observations rather than an invalidation of the comparison since it is the existence of variation - not the source - that is under study.

## Analyses

Initial analysis of PCR data - including setting baselines for background fluorescence, thresholds for calculation of CT scores, and exclusion of samples with unusual amplification or melt curves - was carried out on the AB Step one Plus software. Standard curves were checked to ensure $R^2$ scores greater than 0.99 and efficiency scores in the range 80-120%.

To account for differences in extraction efficiency, virus quantity scores were standardised by dividing by the concentration of DNA in each sample extract. Nucleic acid extractions should be made up of a minimum set of DNA from host and virus and there is no *a priori* reason to suspect that the ratio of virus to host DNA would differ across samples, at least for samples of the same tissue/swab type.
Sensitivity and specificity of tests were compared to livers as this is the tissue that has commonly been used for ranavirus screens, being upheld as the 'gold standard'. Gray et al. (2012) made the same comparison.

The association between pathologies and exposure to ranavirus was tested using Fisher's exact test due to the small numbers sometimes involved.
Dose effect on timing of signs was explored using single factor analysis of variance (ANOVA) with two levels (low and high dose). Dose effect on viral load in different sample types was also analysed using ANOVA but it was necessary to transform the

load data to ensure the data approximated to normal (assessed by eye with reference to QQ plots). Transformation was performed by taking roots: the third root of the throat swab load data, fifth root of the pericloacal swabs, and fourth root of the other sample types.

To assess whether animals that died later had greater pathogen burdens a linear regression of load as a function of day of death was performed.
Alternative sampling protocols were compared to every other protocol studied using pairwise Fisher's Exact tests, again because of the sometimes small numbers involved. All statistical analyses were carried out in R.

# Results

## Summary of mortality

20 animals died during the first 12 hours following the bath exposure; 8 controls, 4 low dose, and 8 high dose. 70 animals therefore went forward into the experiment (22 control, 26 low dose, 22 high dose). All high dose animals died without intervention between days 6 and 10 post-exposure. Eight low dose animals were euthanised at day 8 post-exposure with the remainder dying between days 8 and 20. Just one animal (from the low dose treatment) of those exposed to ranavirus lived past day 12. Eleven of the control animals were euthanised on day 8 and the remaining eleven were euthanised at day 20.

Table 2.1. Incidence of pathologies and abnormalities by treatment and association with ranavirus exposure. All pathologies are included whether observed in live animals or during post-mortem examinations. 'Dose p-values' are for Fisher's Exact Tests. a) incidence varies significantly ($p<0.05$) across treatments b) no significant variation detected across treatments.

a. Signs that are significantly associated with ranavirus exposure

| Treatment | Limb erythema | Lip erythema | Other erythema | Vomit | Blood-tinged vomit | Bleeding | Erythema (pm) | Congested limbs (pm) | Petechial haemorrhages | Ulceration | Inappetence | Muscle abnormal | Tongue abnormal | Mucus - in mouth | Mucus bloody |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control n=22 | 2 | 0 | 2 | 0 | 0 | 0 | 2 | 7 | 1 | 1 | 3 | 0 | 6 | 2 | 2 |
| Low Dose n=26 | 15 | 10 | 10 | 11 | 6 | 8 | 11 | 23 | 12 | 9 | 16 | 7 | 17 | 16 | 12 |
| High Dose n=22 | 15 | 14 | 12 | 12 | 7 | 7 | 8 | 17 | 10 | 11 | 16 | 4 | 11 | 10 | 9 |
| Dose p-value | 7.02E-05 | 6.67E-06 | 0.0037 | 4.11E-05 | 0.0106 | 0.00551 | 0.0282 | 0.000131 | 0.00166 | 0.00177 | 0.000111 | 0.0191 | 0.0343 | 0.000432 | 0.0136 |

b. Signs not significantly associated with ranavirus exposure

| Treatment | Swollen eye | Bloody SC fluid | Fluid in coelom | Bloody fluid in coelom | Liver autolysed | Heart-pale | Lung congestion | Stomach - Diffuse congestion | Stomach - anterior congestion | Intestines - congestion | Gonads congested | Kidneys congested | Abnormal spleen | Sternum - bloody | Skin sloughing |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control n=22 | 0 | 5 | 2 | 2 | 2 | 0 | 12 | 9 | 3 | 7 | 11 | 13 | 1 | 4 | 2 |
| Low Dose n=26 | 3 | 3 | 0 | 0 | 8 | 3 | 9 | 13 | 5 | 13 | 10 | 8 | 5 | 6 | 6 |
| High Dose n=22 | 2 | 0 | 4 | 2 | 5 | 2 | 10 | 8 | 10 | 5 | 9 | 9 | 1 | 1 | 6 |
| Dose p-value | 0.363 | 0.0629 | 0.07 | 0.252 | 0.205 | 0.363 | 0.409 | 0.624 | 0.0385 | 0.143 | 0.755 | 0.135 | 0.206 | 0.179 | 0.321 |

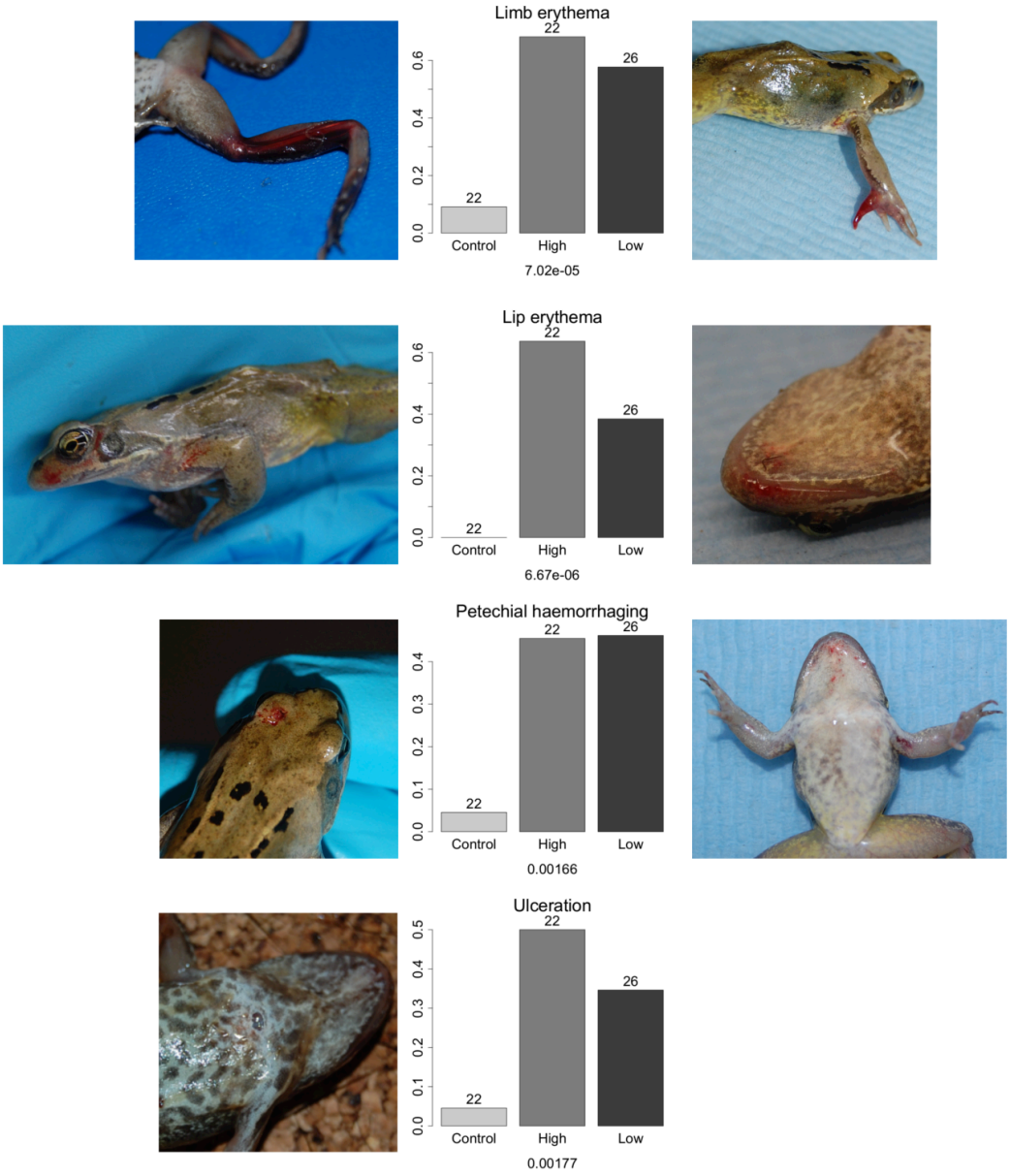


Figure 2.1. Selected signs of ranavirus disease: (top-bottom) erythema of limbs, erythema of lips, petechial haemorrhaging, ulceration. Plots show proportions of animals exhibiting pathology by treatment. Numbers above bars represent total number of animals per treatment. Numbers below plots are p-values from Fisher's exact tests.

## Suspected pathology

Table 2.1 contains suspected pathologies observed across treatments during the experimental period and/or post-mortem examinations. Figure 2.1 summarises data for selected pathologies that are strongly associated ($p<0.002$ in Fisher's exact test) with exposure to ranavirus and includes photographic images of signs.

Signs such as erythema of the lips, regurgitation (with and without blood), bleeding and muscle abnormalities are highly specific - occurring only in animals from the exposed treatments. However, there are other signs (other erythema, petechial haemorrhaging, ulceration, inappetence, mucus in mouth/gut, and tongue abnormalities) that - in spite of their significant association with exposure to ranavirus - were observed at low prevalence in control animals in addition to their higher prevalence in exposed animals.

Incidence of subcutaneous fluid was the only sign significantly associated with euthanasia (Fisher's exact test, $p<0.01$) (see figure 2.2).

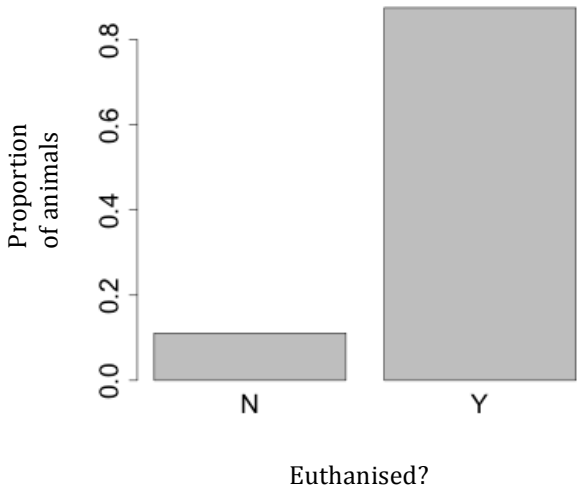


Figure 2.2. The majority of euthanized animals had large amounts of subcutaneous (SC) fluid at post-mortem examination. Plot shows proportion of animals with SC fluid for euthanized (Y) animals compared to animals found dead (N).

## Disease Progression

Disease progression was rapid in both treatment groups exposed to virus. All high dose animals died by day 10 post-exposure; all but one of the non-euthanised low dose animals (18 of the total of 26) had died by day 12.

Clearly, dose impacted on the timing of signs of disease (see figure 2.3). There were significant dose-dependent differences in the timing of four signs of ranavirosis; lip erythema occured on average 1.4 days later in low dose animals compared to high dose ($F=6.763$, $df=1,22$, $p=0.02$), other erythema was 3.2 days later on average in low dose ($F=8.149$, $df=1,20$, $p=0.01$), petechial haemorrhaging in the skin was 3.6 days later ($F=60.97$, $df=1,13$, $p<0.0001$), and regurgitation was 2.9 days later ($F=6.982$, $df=1,21$, $p=0.02$). However, variation in timing for four other signs could not be attributed to dose; innappetence ($F=2.189$, $df=1,12$, $p=0.16$), limb erythema ($F=1.444$, $df=1,28$, $p=0.24$, ulceration ($F=2.492$, $df=1,6$, $p=0.17$) and bleeding ($F=0.0061$, $df=1,12$, $p=0.939$).

For animals in the high dose treatment, ulceration and bleeding were late stage signs of ranavirosis (mean day of occurrence was 10 and 8 respectively) with little difference in timing among other signs (all means are between days 6 and 7) (see figure 2.4). In the low dose treatment, erythema of the limbs and lips were early signs of ranavirosis along with bleeding, whilst erythema in other regions of the skin, petechial haemorrhaging, and ulceration had a later onset.

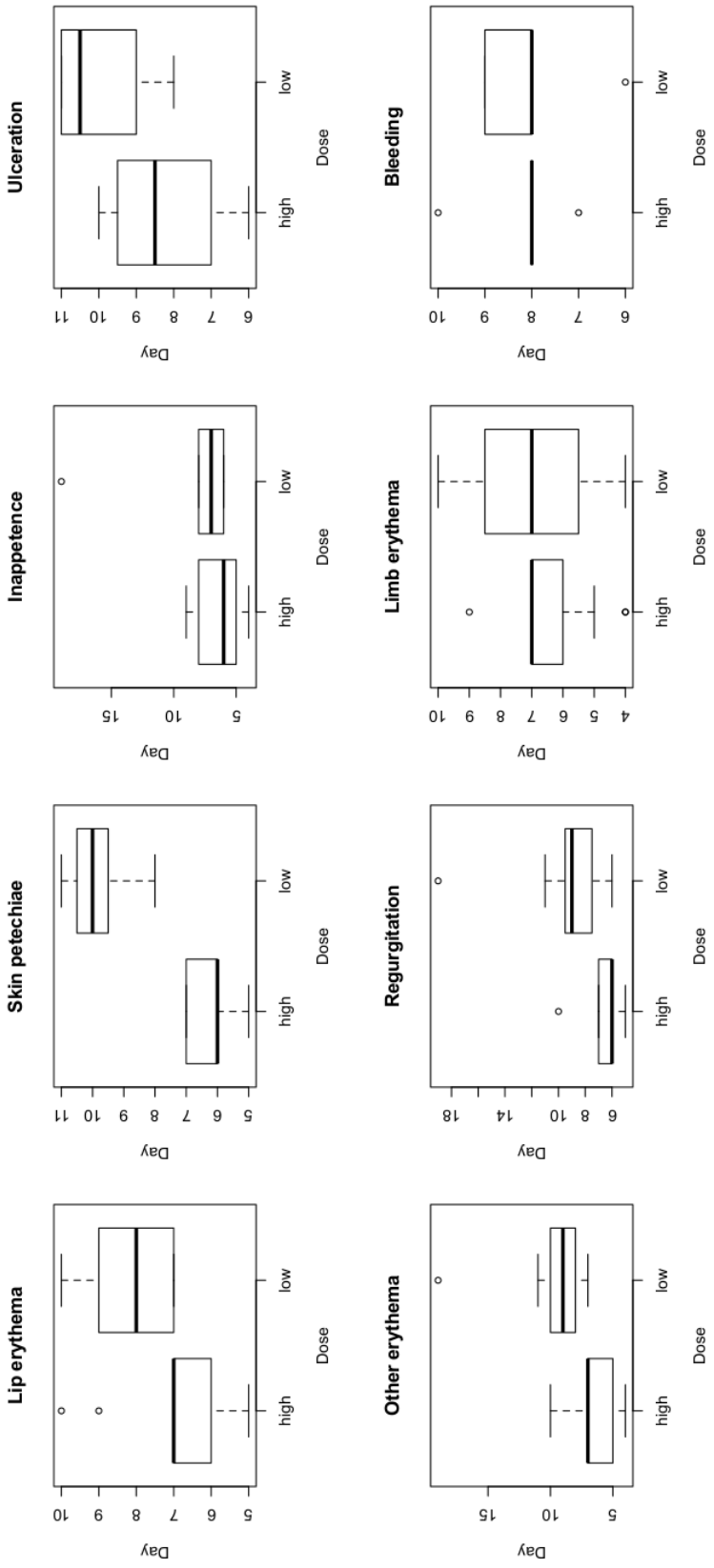


Figure 2.3. Dose effects on the onset of disease by pathology type. Higher concentration inoculate leads to earlier onset for lip and other erythema, skin petechiae and regurgitation but there was no significant difference between high and low dose treatments for other signs of disease.

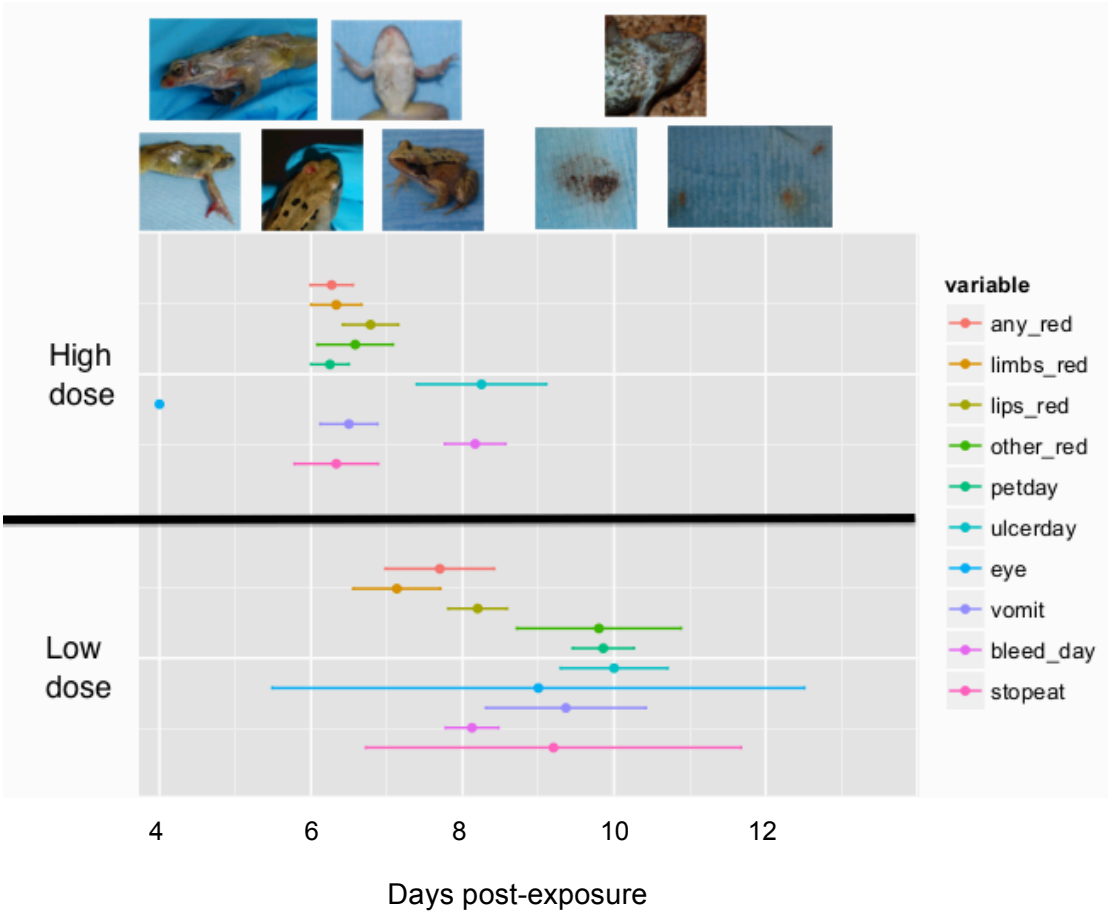


Figure 2.4. disease progression summary showing timing of signs of disease for both high (top) and low dose (bottom) treatments; shows mean day number post exposure +/- SEM.

## Dose effects on viral load

Both types of swab (pericloacal and throat) detected an effect of dose, however the effect for throat swabs was statistically weak (see Table 2.2). No effect of dose on viral load was detected with kidney, liver and toe-clip samples (see figure 2.5).

Table 2.2. Dose effects on viral load by sample type. Summary of analyses of variance tests.

| Sample | F-statistic | df | p |
|---|---|---|---|
| Pericloacal swab | 12.35 | 1,36 | <0.005 |
| Throat swab | 3.76 | 1,34 | 0.061 |
| Kidney | 0.0144 | 1,42 | 0.905 |
| Liver | 0.5943 | 1,43 | 0.445 |
| Toe-clip | 0.6003 | 1,41 | 0.443 |

## Post-mortem viral load versus duration of infection

Post-mortem pathogen burden does not vary significantly according to duration of infection, regardless of tissue sampled:

- kidneys (R-squared=-0.02294, F=0.03581, df=1 and 42, p=0.8508),
- livers (R-squared=0.00584, F=1.258, df=1 and 43, p=0.2682),
- toeclips (R-squared=0.03331, F=2.447, df=1 and 41, p=0.1254).

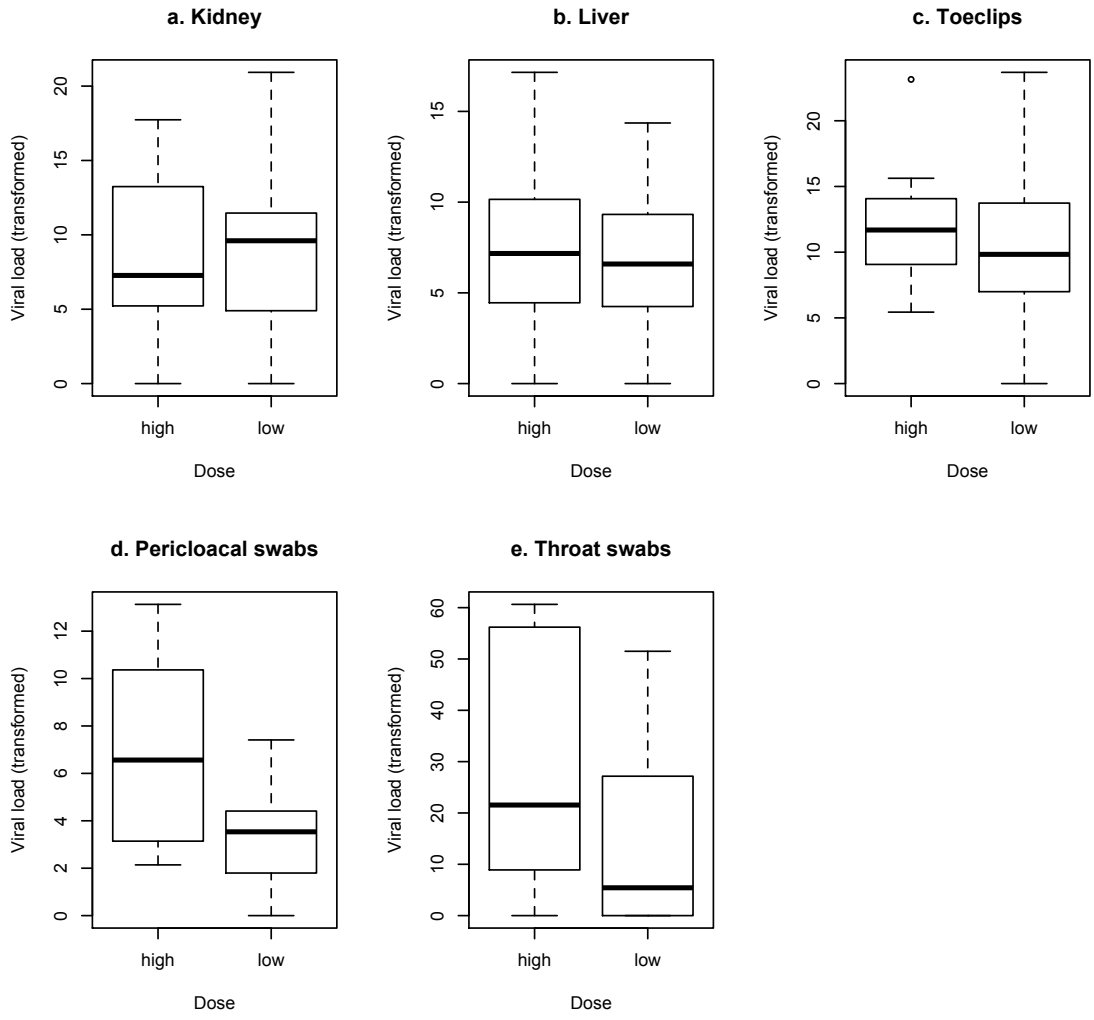


Figure 2.5. Effect of dose on viral load by sample type (a-e). Swab samples were taken from live animals on days 8 or 9 post-exposure whilst tissue biopsies were taken post-mortem.

## Ranavirus screening

Ranavirus screens yielded many positive results across all sample types; 61% of kidneys screened were positive, 63% of livers, 65% of toeclips, 56% of pericloacal swabs, and 47% of throat swabs.

Ten pairwise comparisons of sample types were made with both sample types yielding positives not revealed by the other in all but three of the comparisons. These three all involved toeclips with none of kidney, liver or PC swab yielding any positives that were not also identified by toeclips. One low dose animal - euthanised at day 8 - was not found positive by any of the sample types despite showing a sign (limb erythema) found

to be consistent with ranavirosis. Three samples across all sample types from control animals (all throat swabs) screened positive for ranavirus.

There were significant associations ($p<0.0005$) between all of the methods used (see Table 2.3) in spite of differences in performance between sample types (see figure 2.6). There was little variation in specificity across sample types but sensitivity did vary:

- More infections were detected from toeclips than from any of the other samples types
- Livers had lower sensitivity than toeclips
- Kidney samples detected more infections than 2 of the other samples types (PC and throat swabs; with notably higher sensitivity than throat swabs) and less for the other two (livers and toeclips)
- PC swabs were more sensitive than throat swabs only
- Throat swabs (as well as yielding 3 false positives) were less sensitive than all of the other sample types.

Table 2.3. Associations between sample types in ranavirus screening. P-values from pairwise comparisons of sample types using Fisher's Exact tests.

| | | | | |
|---|---|---|---|---|
| Liver | $7 \times 10^{-12}$ | | | |
| Toeclip | $7 \times 10^{-14}$ | $5 \times 10^{-15}$ | | |
| PC swab | $2 \times 10^{-10}$ | $4 \times 10^{-11}$ | $6 \times 10^{-12}$ | |
| Throat swab | 0.0001 | 0.0001 | $9 \times 10^{-6}$ | $2 \times 10^{-6}$ |
| Sample type | Kidney | Liver | Toe | PC swab |

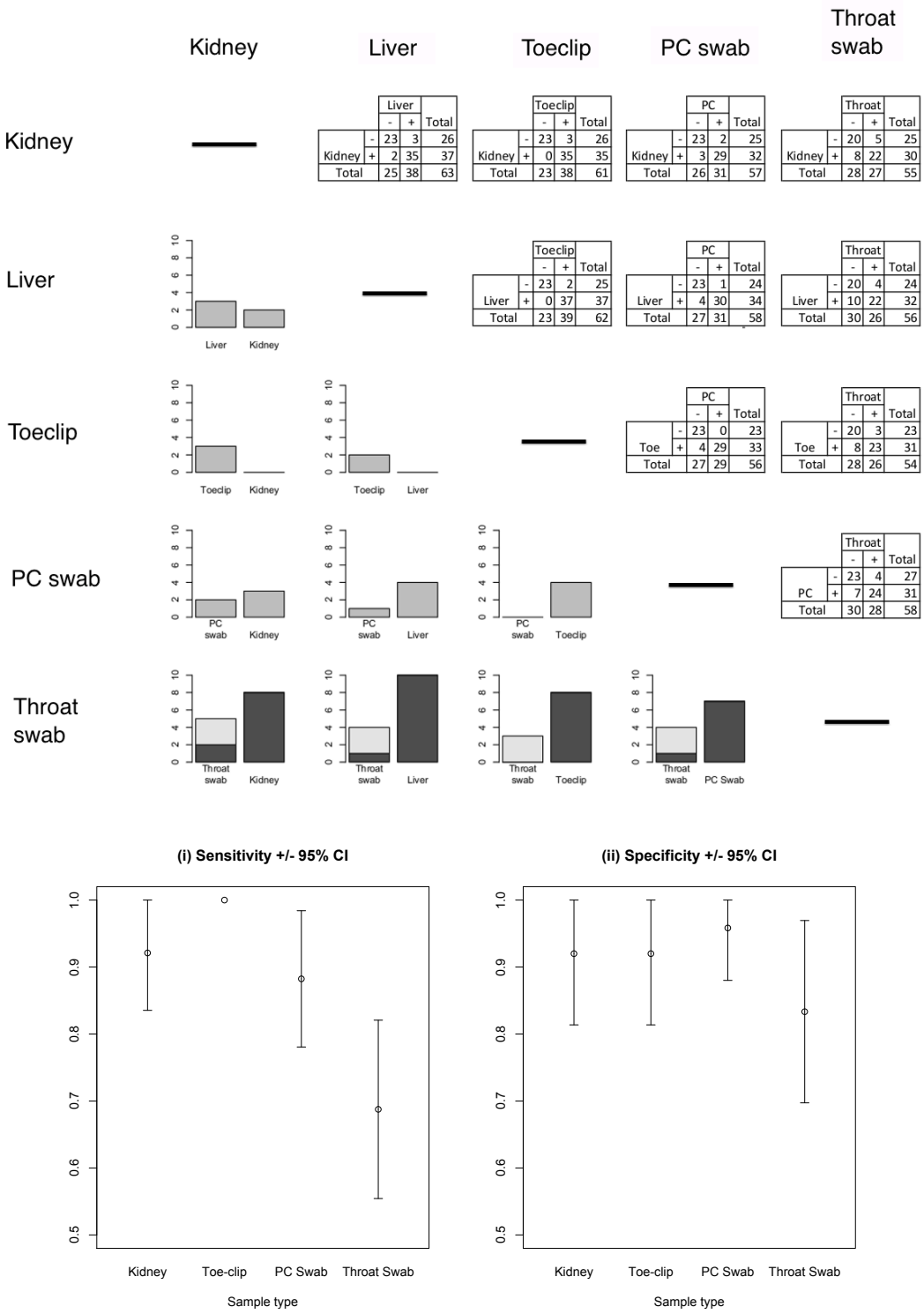


Figure 2.6. Performance of sample types in ranavirus screening. Top; Pairwise comparisons of tissues (kidney, liver, toeclip) and swabs (pericloacal (PC), throat); contingency tables and barplots showing the number of additional positives not detected by the compared sample type. Light fill in throat swab plots indicates false positives. Bottom) (i) Sensitivity and (ii) Specificity by sample type with reference to liver samples; bars show 95% confidence interval.

# Discussion

Common frog populations faced with persistent disease can experience greater declines within larger populations (Teacher et al., 2010), suggesting an effect of density on transmission. Results from the swab samples in this study show that increasing the initial concentration of virus that a host is exposed to can lead to a significant increase in the amount of virus being shed at a given point later on in the disease process. This assumes that the amount of virus detected by an external swab is proportional to the total amount of virus being released into the environment. Infections in large populations will then set off a positive feedback loop whereby individuals are exposed to high initial doses and quickly get to a point in the disease process where they are shedding high concentrations of virus and adding to the high dose pool that other individuals are exposed to. Teacher et al. (2010) also found that populations rarely went extinct, but instead persist at low densities, maintaining infections in the population. An alternative explanation of this pattern of recurrent disease is that immigrant frogs from the surrounding area arrive at these ponds after mortality events and serve as a new cohort of susceptibles.

This experiment revealed other dose effects. Lower initial exposure prolonged the time-course of disease and death and introduced much more individual variation in timing of onset of signs of disease. In addition, pathogen burden was linked to initial exposure prior to death, but at death this was not the case. Rather, viral replication and dispersal through most tissues seems to occur until host tolerance is exceeded at some "critical threshold".

## Ranavirus sampling

Lethal, non-lethal and non-invasive sampling strategies for ranavirus were compared in this study. All sampling strategies performed quite well, detecting the majority of ranavirus infections and being associated statistically. Toe-clips outperformed all other sample types for sensitivity, adding to the work of St. Amour and Lesbarreres (2007) in showing their utility. Toe clipping can be considered a non-lethal sampling strategy since animals do not need to be euthanized and removed from the wild. However, given license restrictions, toe-clip samples were taken post-mortem in this experiment. Other data presented here shows that viral load

increases until death meaning that my toe-clipping routine sampled animals at peak viremia and it's likely that when toe clipping is used as a truly non-lethal strategy then the detected load - and hence, the method's sensitivity - would be reduced. The detection of false positives among throat swabs indicated problems with contamination or specificity and throat swabs were also the least sensitive strategy. Pericloacal swabs showed increased sensitivity and specificity compared to throat swabs and seem good candidates for screening live animals. As mentioned above for toe-clips, peak viraemia seems to occur at time of death, so the sampling of live animals at an earlier stage in the pathogen population growth curve might explain some or all of the small differences in sensitivity compared to visceral organs.

## Ranavirus consistent signs and disease progression

There were fifteen suspected pathologies measured in this study that had a very strong association with ranavirus exposure and are therefore taken to be signs associated with ranavirosis. The most consistent pathologies as diagnostic signs were erythema of the lips, regurgitation, bleeding and muscle abnormalities. Unfortunately bleeding could be mistaken for predation wounds by an untrained observer, citizen scientists are unlikely to ever see any muscle tissue and witnessing a regurgitating amphibian would require obsessive dedication to the cause. Ulceration (and associated digit necrosis), erythema and petechiae are therefore likely to be the most useful for citizen-science led surveillance of the remaining positively associated pathologies since they are overt, consistent signs.

I also compared the incidence of pathologies in euthanised versus non-euthanised animals in the low dose treatment as above. There were a number of significant ($p<0.05$) contrasts in the proportion of affected animals - limb and other erythema, regurgitation, tongue abnormalities, and subcutaneous fluid. All except subcutaneous fluid were strongly associated with ranavirus exposure (as explained above), had lower incidence in euthanised than non-euthanised animals, and can be explained by disease progression in euthanised animals not being far enough advanced for these signs to be showing at the time of euthanasia. The association between subcutaneous fluid and euthanised animals is not surprising given that these animals were immersed in a concentrated anaesthetic solution for 3 hours prior to post-mortem examination.

The initial pathological investigation of unusual amphibian mortality in the UK found evidence for two disease syndromes (ulcerative [US] or haemorrhagic [HS]) (Cunningham et al., 1996). As a consequence of the rapid mortality observed here and the discrete nature of the observation periods (once per day), signs of disease tended to be observed in clusters, which were hard to distinguish temporally (see figure 2.4). This is true for both ranavirus treatments but high dose animals showed very little variation among individuals in timing of particular signs, especially when compared to low dose animals. There were some indications of disease progression however; ulceration had a significantly later time of onset than erythema, petechial haemorrhaging and other signs in high dose animals. This suggests that these signs are part of a single disease continuum. This possibility has been considered previously, and some support for this point of view is the poorer body condition and increased emaciation among animals exhibiting ulceration without haemorrhaging (Cunningham et al., 1996) and the presence of viral antigen in the dermis and epidermis of animals with both syndromes (Cunningham et al., 2008).

Attempts to reproduce the naturally observed syndromes in the laboratory by exposing animals to tissue homogenates produced from tissues of diseased animals at sites where only HS or only US was evident (Cunningham et al., 2007) were partially undermined by weaknesses in experimental design including small sample size and a failure to equalise virus titres across treatments with the possibility that exposure to less concentrated inoculate enables a more effective immune response to be mounted and limits incidence of systemic disease. As in this study, when exposing frogs to virus cultured from tissues of hosts with US, Cunningham et al. (2007) found that animals suffered acute disease and exhibited signs consistent with both syndromes. The fact that two disease 'syndromes' can appear within a disease progression continuum suggests that the reported compartmentalisation of syndromes at ponds may result from ecological factors relating to pond management or situation (e.g. pesticide use).

Cunningham et al. (2007) also compared the effects of exposure to tissue homogenate and cultured virus directly. They found that cultured virus increased the number of animals with systemic disease and decreased the time to death. They suggest that either selection in culture altering the make up of heterogeneous virus populations, or cell culture mediated effects on gene expression could explain this. Indeed, passage in

cell culture could quickly select for virions that enter cells and replicate rapidly which could act on the genetic variation within a virus population (Ebert, 1998), but could also alter the ratio of enveloped to naked virions, a factor known to affect cell entry (Chinchar, 2002). DNA viruses have traditionally been considered to evolve much more slowly than RNA viruses and speciate alongside their hosts, but recent work has shown that mutation rates can approach those of RNA viruses (Firth et al., 2010) and reinforces Cunningham et al.'s (2007) suggestion that the different outcomes in homogenate versus cultured virus treatment could be driven by selection of virus types within a heterogeneous population during cell culture passage. Whilst placing question marks over the biological relevance of attempts to use cultured virus to study the disease process it does raise interesting questions and warrants further studies into the effects of ongoing passage on infectivity and virulence (especially since the culture process may mimic possible effects of aquaculture on pathogen virulence) as well as next-generation sequencing strategies to characterise virus heterogeneity in diseased animals.


chapter three
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# 3

# Humans as drivers of an emerging infectious disease of wildlife: spatial epidemiology of UK ranavirus disease

## Abstract

Humans are frequently implicated in the spread of emerging infectious diseases (EIDs) of wildlife. Emergence may result from specific human interactions such as the translocation of infectious organisms yielding novel pathogens or from indirect

interactions with the environment such as climate change, which may alter the evolutionary or ecological equilibrium between an endemic pathogen and its host(s).

Here I use a large database of unusual frog mortality events to separate reports consistent with ranavirus infection and model spread of disease in the UK. I compare climate change and translocation of animals by humans as hypotheses explaining spread. Spatio-temporal models consisting of endemic (imported) and epidemic (self-exciting) components are implemented within the R package, Surveillance.

All models are improved by including a self-exciting epidemic component, demonstrating that ranavirosis is spreading and that new events are not merely an artifact of reporting effort (which is not clear from initial visualization of the data). Human population density is a more powerful explanatory variable than temperature in modeling spread but both covariates help to explain the observed point pattern and contribute to the best-performing model. Infectivity drops off steeply with distance from sites of primary infection with few secondary infections more than 2km away from the parent event. Imported infections drive spread until 1997, after which time the majority of new events are explained by transmission between ponds.

These models offer further evidence of the link between human activity and disease emergence though it is not clear if this is due to translocation, habitat disruption, or another consequence of human activity. The analyses also follow up experimental observations of the effect of temperature on ranaviruses and suggest that these growth preferences may have had a real impact on pathogen establishment during emergence.

# Introduction

Emerging infectious diseases of wildlife (EIDs) have been split into three categories according to the degree of human intervention: those spread 1) by spillover of infection from domestic animals, 2) by human translocations of pathogen or host, and 3) through no direct human intervention (e.g. triggered by climate change) (Daszak, 2000). Two hypotheses can be considered to explain the emergent nature of a novel epidemic: the novel pathogen hypothesis (NPH) and the endemic pathogen hypothesis (Rachowicz et al., 2005). The NPH describes an invasive pathogen, often spreading from an unidentified source, whereas the EPH details an altered relationship between host and pathogen driven by environmental change (Garner et al., 2012).

Both of these possibilities might explain the apparent emergence of a ranavirus in the UK although most accounts concentrate on the NPH; this interpretation has been encouraged by genetic analysis, which at first sight points to an introduction from North America prior to 1985 (Cunningham et al., 1996; Hyatt et al., 2000). Mortality consistent with the pathogen began to be reported after this date; infections are known to be now widespread in England (Cunningham, 2001) and some populations experienced dramatic declines between the mid-90s and 2008 (Teacher et al., 2010). The genetic evidence for introduction is, however, based on limited sequence data from a highly conserved gene, and no other techniques have been used to study emergence of ranavirosis in the UK. In this chapter I analyze the records of disease spread to obtain a separate line of evidence.

## Wildlife translocations

International trade in ornamental fish species, and in amphibians for food and as pets is discussed in chapter 4 in the context of ranavirus translocations across borders and between continents. Although the UK has not participated in the massive imports and exports of amphibians for food, the UK public have been drawn to the purchase of exotic amphibians as pets since the second half of the 20$^{th}$ century (Langton et al., 2011) and there are between 2.5 and 3.5 million domestic garden ponds in the UK which are commonly stocked with ornamental fish (Schloegel et al., 2009). The presence of ranaviruses among traded animals is therefore a potential explanation for the spread of ranaviruses at a national scale, and offers a mechanism that would explain how a single introduction event could have led to widespread national and regional dispersal through the import of contaminated stock.

Some species, for example bullfrog (*Lithobates catesbeiana*) and the African clawed frog (*Xenopus laevis*), can carry asymptomatic infections and could therefore be candidates for vectors of the virus without drawing attention to the danger by overt signs of disease or poor condition which would decrease their value or chance of sale. Such traded exotic amphibian species, though they have been classed as invasive, do not have extensive contact with native amphibian populations – being restricted to just a small number of ponds and only occasionally establishing breeding populations. Their distribution has remained restricted, occasionally because of active management, as is

the case for the North American bullfrog (Langton et al., 2011). Nevertheless, the occasional release of individual animals could still initiate disease outbreaks even if conditions are unsuitable for their survival. An example of the potential importance of human translocations is provided by the case of ranavirus-spread among salamanders in North America (Jancovich et al., 2005; Picco and Collins, 2008). In this case the virus is thought to have been spread in juvenile salamanders ('waterdogs'), which are routinely sold to anglers as fishing bait, with unused animals frequently being released after fishing expeditions (Picco and Collins, 2008).

A comparable practice, that could have spread pathogens in the UK, is the widespread practice of 'spawn swapping': the translocation of spawn between ponds was seen as a way to increase amphibian distribution and diversity, until recent years when it was actively discouraged via a national campaign in the media (e.g. See http://news.bbc.co.uk/1/hi/uk/7282649.stm). Genetic evidence suggests that such translocations have even taken place between England and Ireland, backed up by subsequent interviews with pond owners (Teacher et al., 2009b).

## Climate

The alternative class of explanation for the appearance of ranavirus, the endemic pathogen hypothesis (EPH), is some influence of climate on disease and infection dynamics. Such effects could be mediated through effects of climate on host defense, pathogen abundance and virulence. Evidence that such a causal link is plausible comes from studies showing that temperature variation affects amphibian immunity and can lead to increased susceptibility to infectious disease (Raffel et al., 2006). These studies were on individuals, but there is evidence at the population level that immunity is optimised through coevolution of host and pathogen within a particular range of temperatures; and that the results can be sub-optimal outside of this range (Fisher, 2007). This type of evolutionary dynamics may explain why warmer years (including milder winters) between 1983 and 2005 appear to have had a negative impact on a common species of toad (*Bufo bufo*) in the UK (Reading, 2007). Climate change can also alter amphibian behaviour such as timing and duration of hibernation (Reading, 1998), which may affect pathogen transmission opportunities.

Ranaviruses may be affected by such temperature-dependent effects: they exhibit temperature sensitivity both in the wild and in the laboratory. When grown at controlled temperatures in cell culture, replication is more rapid at higher temperatures, with an optimum at 24ºC (Ariel et al., 2009). Although it is problematic to extrapolate directly from laboratory studies to ecology in the wild, such results indicate that climate change could alter the geographical realisation of the virus's fundamental temperature niche, and ultimately the spatial distribution. In the wild ranavirus outbreaks show marked seasonality - usually occurring in the summer months - but Gray et al. (2009) note that this may be more of an issue of visibility rather than actual incidence. Increasing temperatures are also thought to remove a key check on pathogen abundance since milder winters could result in greater overwintering success of pathogens (Harvell et al., 2002).

The Frog Mortality Project in the UK was established because of increasing number of reports of unusual mortality and has been compiling them in an impressive citizen science campaign, which has provided a long-term dataset, and which I analyse here. The FMP database contains large amounts of data including information about the signs or pathologies studied in chapter 2. Filtering the dataset based on pathologies consistent with ranavirus infection enabled me to model climate and translocation as alternative hypotheses that may drive emergence and spread of ranavirus infection and disease.

# Methods

## FMP reporting

Froglife (registered charity number 1093372 in England & Wales) have received and collated reports of unusual amphibian mortality from the public since 1992 (Cunningham, 2001). Reports were solicited through nationwide appeals via all forms of the popular media - annually until 1997 and intermittently since then (Cunningham, 2001). Reports were filed as hard copies until 2001 and a mixture of electronic and paper afterwards. A major round of data entry retrospectively consolidated the FMP as a relational database, which was used for this analysis. Access to an online electronic report form is now unrestricted. Previously paper reports were issued on request and usually after correspondence about the nature of the mortality with events

not obviously consistent with ranavirosis sometimes not pursued further. The change in the method of data acquisition may therefore have changed the frequency of reports and the overall proportion of non-ranavirus related reports submitted. Would-be reporters are still filtered according to Froglife's description of a ranavirus-consistent mortality event by phone and email (personal observation) and via text on their website (Froglife, http://www.froglife.org/disease/report.htm, accessed 10/09/13) with some reporters discouraged from filing a report.

## FMP database manipulation

### Georeferences

Postcodes were included with reports and were used to provide approximate geographical positions of mortality events. Postcodes are discrete data but they cover small areas (typically 15 households, http://cdu.mimas.ac.uk/pclut/) in the context of analyses at a national scale and are considered as continuous spatial references in this study. Each postcode was converted to OS National Grid References (EPSG, 27700), a Government Office Region (GOR) code, and an urban/rural indicator code using GeoConvert Postcode Data for 2006 onwards form the National Statistics Postcode Directories (http://geoconvert.mimas.ac.uk, accessed 10/09/13).

### Timing of mortality events

The precision of timestamp information in the FMP database in relation to onset and duration of mortality events is usually only at the level of month. There is also uncertainty about the timing of transmission and the onset of disease: ranavirus outbreaks are focused in adults in the UK (Duffus, 2009) and have a peak incidence in summer months (Cunningham, 2001). Animals can be infected via a variety of routes in the laboratory (Cunningham et al., 2007) but - given the observed infection dynamics, phenology of the *R. temporaria* life cycle, and potential for amphibian immunity to vary seasonally - transmission by direct contact during the breeding season and a lag in disease onset can be considered a likely route in the wild (Brunner et al., 2007). For these reasons it was considered that an outbreak could be classified to the year in which it occurred, but no finer resolution. Because the analysis software does not automatically deal appropriately with multiple observations with identical times, unique times for each event were generated by randomly drawing a time from within the year,

following the recommendation of one of the authors of the package used in the analysis (Meyer, pers. comm.).

The reports were filtered for consistency with ranavirus infection. "Ulceration", "red spots on body" and "limb necrosis/loss of digits" were the signs of disease chosen to reliably represent ranavirosis (see Cunningham (2001) and chapter 2). In addition, reports were only considered ranavirus consistent if there were at least five animals involved in the mortality event given the virulence and infectivity of the virus.

## Covariate data

Climate and population data was obtained to parameterise alternative models of the spread of ranavirosis.

Table 3.1. Location, altitude and name of UK weather stations with long term climate records used for climate covariate

| Region | Station Name | OS easting | OS northing | Altitude (m) |
|---|---|---|---|---|
| North-West | Newton Rigg | 3493E | 5308N | 169 |
| East of England | Cambridge NIAB | 5435E | 2606N | 26 |
| East Midlands | Sutton Bonnington | 4507E | 3259N | 48 |
| London | Heathrow | 5078E | 1767N | 25 |
| Northern Ireland | Armagh | 2878E (Irish grid) | 3458N (Irish grid) | 62 |
| North-East | Durham | 4267E | 5415N | 102 |
| Scotland | Braemar | 3152E | 7914N | 339 |
| South-East | Eastbourne | 5611E | 983N | 7 |
| South-West | Yeovilton | 3551E | 1232N | 20 |
| Wales | Cwmystwyth | 2773E | 2749N | 301 |
| West Midlands | Shawbury | 3552E | 3221N | 72 |
| Yorkshire & the Humber | Sheffield | 4339E | 3872N | 131 |

Climate data was downloaded from the Met Office - http://www.metoffice.gov.uk/climate/uk/stationdata/ - where historic station data are freely available. A range of data is available but Mean daily maximum temperature (tmax) was considered the most suitable variable to use in models given the possibility for increased ranavirus replication with increasing temperature (see Introduction). Datasets went back to at least 1964 from all stations used and therefore extended sufficiently far into the past to include our period of interest (reports in the FMP database span from 1991 to 2010). Details of regional stations are in table 3.1 and their distribution with reference to regional boundaries is visualised in figure 3.1.

Regional human population sizes and densities were obtained from the Population Estimates Unit of the Office for National Statistics.

Figure 3.1. Map of UK national and Government Office Region (GOR) boundaries with positions of regional weather stations listed in Table 3.1.

## Two component spatio-temporal models

I used "twinstim" (Meyer, 2010; Meyer et al., 2012), a function in the R package Surveillance v1.5-4, to analyse the UK spread of ranavirus-consistent mortality events. Outbreaks are modeled as Poisson events. The conditional intensity function (CIF) is the instantaneous rate or hazard for events at time, t, and location, s, conditioned on the history of all observations up to time, t, (Meyer et al., 2012) and is the sum of two components – the endemic and epidemic components (see equation 1).

Equation 1:

$$\lambda^*(t, s) = h(t, s) + e^*(t, s)$$

The endemic component represents "imported" cases – arising from unseen sources of infection (or from sources that are uncorrelated with other outbreaks). This component includes a known spatiotemporal intensity offset – here, I use the 'at risk' population of

amphibian ponds having controlled for reporting effort – such that the endemic rate of infection is proportional to the availability of ponds occupied by susceptible amphibians. This component can also include covariates that predict the incidence of imported events. By use of different covariates, I explored the evidence for two alternative hypotheses for spread of UK ranavirosis: Human translocation of virus modeled using human population density as an endemic covariate, and climate change effects on host or virus modeled using temperature as an endemic covariate.

In the current analyses, the epidemic component of infection risk can be thought of as spread from pond to pond mediated via amphibian dispersal. This is the self-exciting component and describes the infection pressure at a given time and location caused by the contributions of each ranavirus infected pond. The contribution an individual event makes to the epidemic component is factorized into the separate effects of 'marks', which may affect the infectivity of an individual event (e.g. fragmentation/continuity of the landscape), the amount of elapsed time since onset of infectiousness, and the location relative to other events. Interaction functions describe the decay of infectivity with distance (siaf) and time (tiaf) from the infection source. I used an 'Urban/Rural Index' as a mark to allow for different infection probabilities across different terrains through the distinction of urban from rural locations. The Urban/Rural Index comprised eight levels, which encoded varying degrees of urbanization from heavily urbanized to sparsely populated hamlets and dwellings.

It should be recognized that the human translocation versus climate change hypotheses are difficult to evaluate directly, but preliminary evidence can be obtained in the spirit of data-exploration: if human translocations were a major source of infection, the risk might be an increasing function of human population density. Hence the effect of that covariate was explored. On the other hand climate change would be expected to result in a relationship with the covariates summarizing the climatic data.

The values of the covariates were obtained at the resolution of government office region (GOR, see figure 3.1) for 9 regions of England plus the national regions of Scotland, Wales and Northern Ireland. Covariate data were split by year and by GOR.

In order to assign sites to GORs, boundary coordinate data for the UK were read into R as a shapefile. The coordinates of polygons representing the British mainland, the Isle of Wight and Northern Ireland were extracted and the polygon boundaries simplified using the function 'dp' from the R package, Shapefiles 0.7. These 3 polygons were then combined in a spatial polygons object, suitable for use with twinstim. The coordinate reference system (CRS) used was The European Terrestrial Reference System 1989 (ETRS89, EPSG 3035) in kilometre units. The locations of ranavirus consistent reports were converted to the same coordinate reference system and plotted to check if they fitted within the modified boundary. Coordinates of the polygon were edited manually where points did lie outside of the simplified boundary.

## Model parameterization

Upper limits for the infectivity of events were set based on knowledge of the biology of frogs: the spatial limit for any pond to transmit infection (eps.s) was set at 30km and temporal limit (eps.t) was set at 2920 days (an approximation of the maximum lifespan of a wild common frog). Human population density and average daily maximum temperature were the variables used in formulating the endemic components in the two competing models of spread.

Table 3.2. Summary of twinstim model parameterisation of models for competing hypotheses ('Translocation' versus 'Climate') explaining UK spread. Alternative covariates (At risk) were used to control for reporting effort and represent the underlying at risk population density in terms of ponds with *Rana temporaria* present. Siaf and Tiaf are spatial and temporal interaction functions describing how infectivity of a primary infection decays in space and time.

| Model type | Endemic | | Epidemic | | Siaf | Tiaf |
|---|---|---|---|---|---|---|
| | At risk | Other | At risk | Other | | |
| Translocation | vrisk10, vrisk50, adjrep10, adjrep50 | Population | vrisk10, vrisk50, adjrep10, adjrep50 | Urban/Rural Index | Gaussian, Constant | Exponential, Constant |
| Climate | | Average maximum daily temperature | | | | |

## Controlling for reporting effort: estimating the at risk population

A covariate was used in order to allow for differences in reporting effort and changes in reporting methodology, and the different density of populations at risk. I reasoned that the effort was proportional to the number of reports of mortality events that were not ranavirus-related (mapped in Figure 3.2). The number of these events, Nn, was therefore included as an offset (transformed as log(Nn+1) since the Poisson model of events has a log link-function).

It was possible that some reporting biases are not compensated for in this manner. For example a campaign of focused soliciting for disease reports took place in London and the South-East (particularly in the early 1990s), and there were other local and national media campaigns (see Cunningham, 2001). Alternatively, filtering of reporters based on particular characteristics of the amphibian mortality events (as discussed above in FMP reporting) could have introduced further biases into the reporting rate.

To attempt to control for these biases I generated 4 alternative variables to represent the underlying pattern of the population at risk (ponds with susceptible common frog populations). The occurrence of ranavirus driven mortality events in a region could bias the number of negative reports received in either direction: i) by "sensitising" citizens of the region through word-of-mouth and local press coverage leading to an elevation in reporting effort which may affect numbers of negative and positive reports, ii) by shifting pond-owners with a keen interest in their pond who might ordinarily have been conscientious reporters of some non-ranavirus consistent mortality event into the positive reporters and thereby decreasing the number of negative reports.

To model these possibilities I created variates vrisk10 and vrisk50 deducted 10% and 50% respectively of the number of positive reports from the number of negative reports for each year by region position in an array. Similarly adjrep10 and adjrep50 added 10% and 50% respectively of the number of positive reports to the number of negative reports for each year by region position in an array.

# Results

## Ranavirus consistent reports

Filtering the database for reports consistent with ranavirosis produced a positive set of 1450 reports (33%) out of the 4458 total reports remaining after database had been purged of reports with essential data missing (e.g. georeference data), obvious inconsistencies and errors. Report numbers - both positive and total - are concentrated in particular years and regions (e.g. 11% of total reports were received in 1995 from the South-East region).

## Visualising epizootic data

Report data are visualised in figure 3.2, which shows time series of the changing distribution of reports - both those consistent with ranavirosis and those that are not. Both types of report appear to increase in abundance and spatial spread over time.

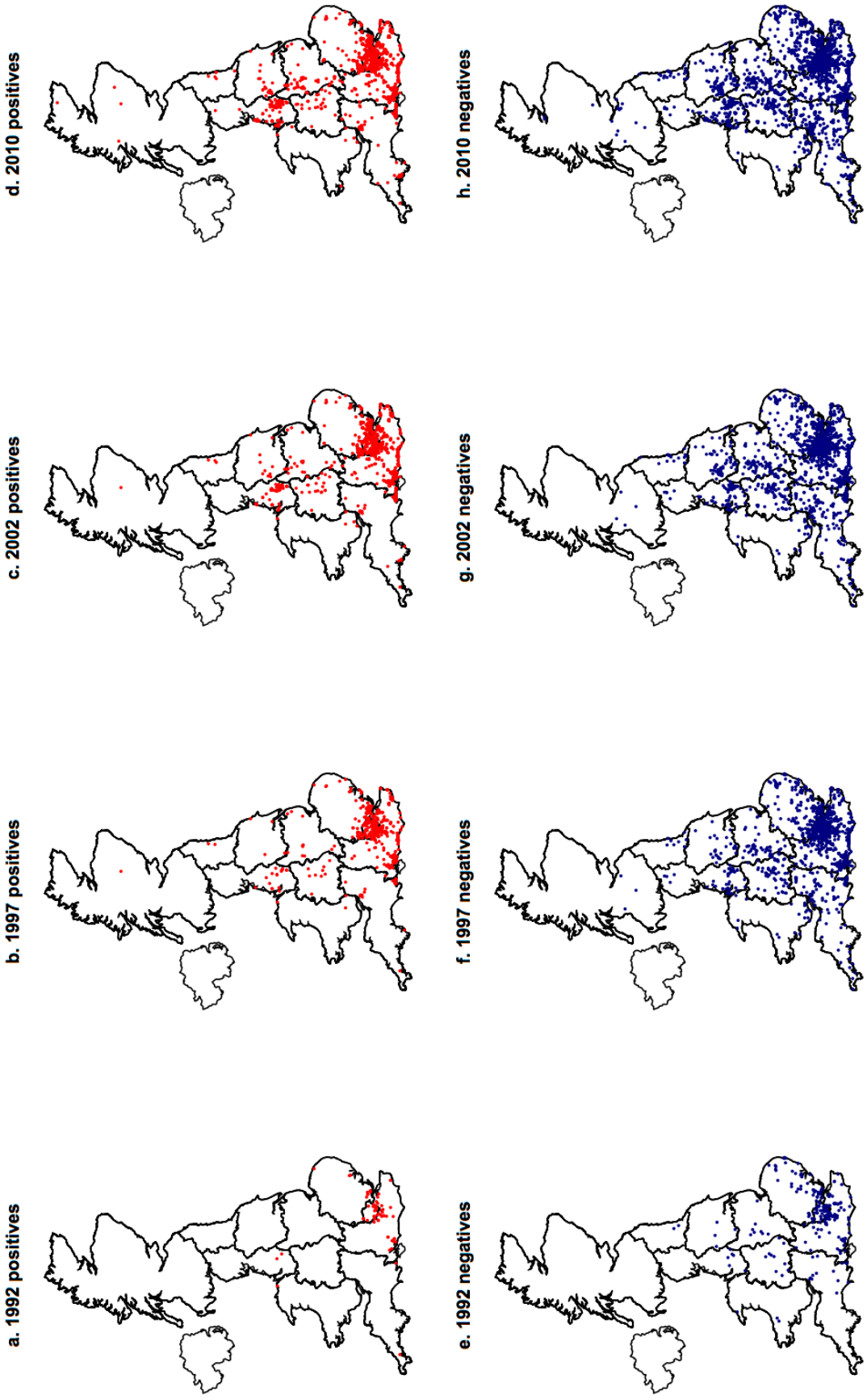

d. 2010 positives
h. 2010 negatives
c. 2002 positives
g. 2002 negatives
b. 1997 positives
f. 1997 negatives
a. 1992 positives
e. 1992 negatives

Figure 3.2. Visualisation of UK spread of ranavirus-consistent mortality after filtering the Frog Mortality Project database of reports of unusual frog mortality. The presence of "Ulceration", "red spots on body" or "limb necrosis/loss of digits" among at least five dead common frogs deemed the event as ranavirus consistent ('positive'). a) - d) show a time series of positive reports (1992, 1997, 2002, 2010), e) - h) show distribution of negative reports at the same timepoints.

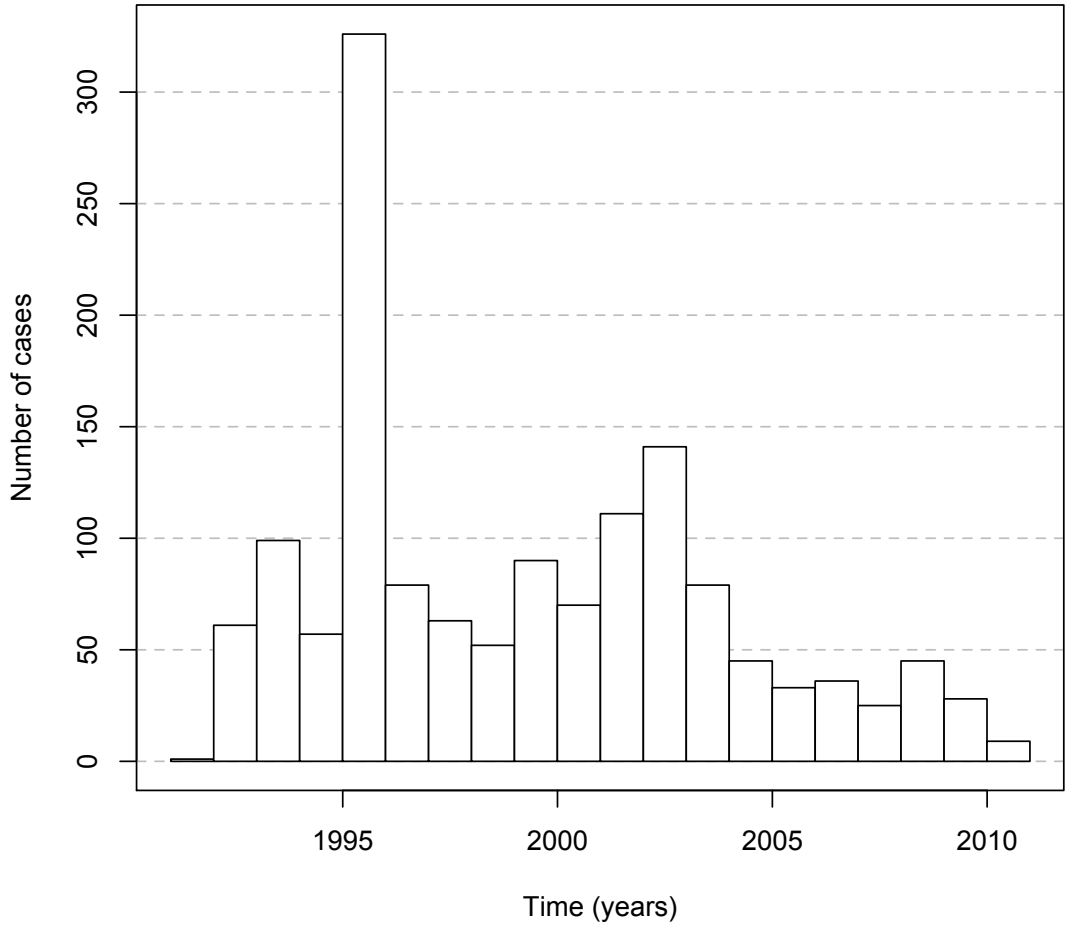



Figure 3.3. Numbers of ranavirus-consistent reports received by the Frog Mortality Project through time from 1991-2010.

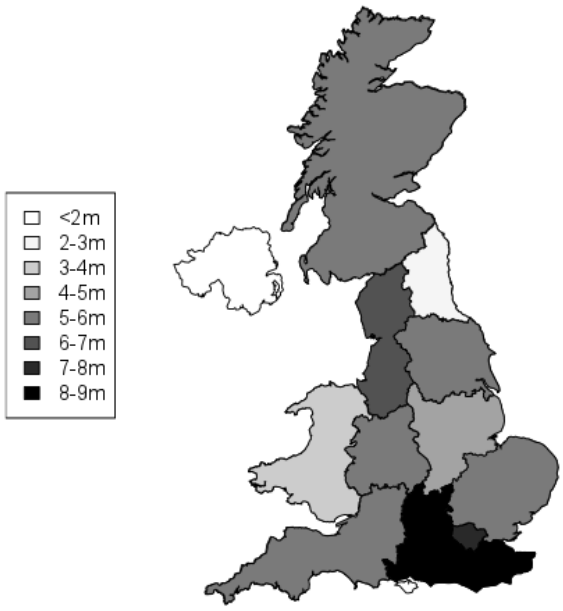



Figure 3.4. UK human population by region (Scotland, Wales, Northern Ireland and Government Office Regions (GOR) of England)

## Evidence for broad scale climate change

Linear regression revealed an increasing trend in temperatures in most regions of the UK between 1991 and 2010 (see figure 3.5). However, there were only significant warming trends in two of the regions: the South-East ($R^2$=0.31, F=8.16, df=1, 18, and p=0.01) and Northern Ireland ($R^2$=0.26, F=6.41, df=1, 18, and p=0.02).

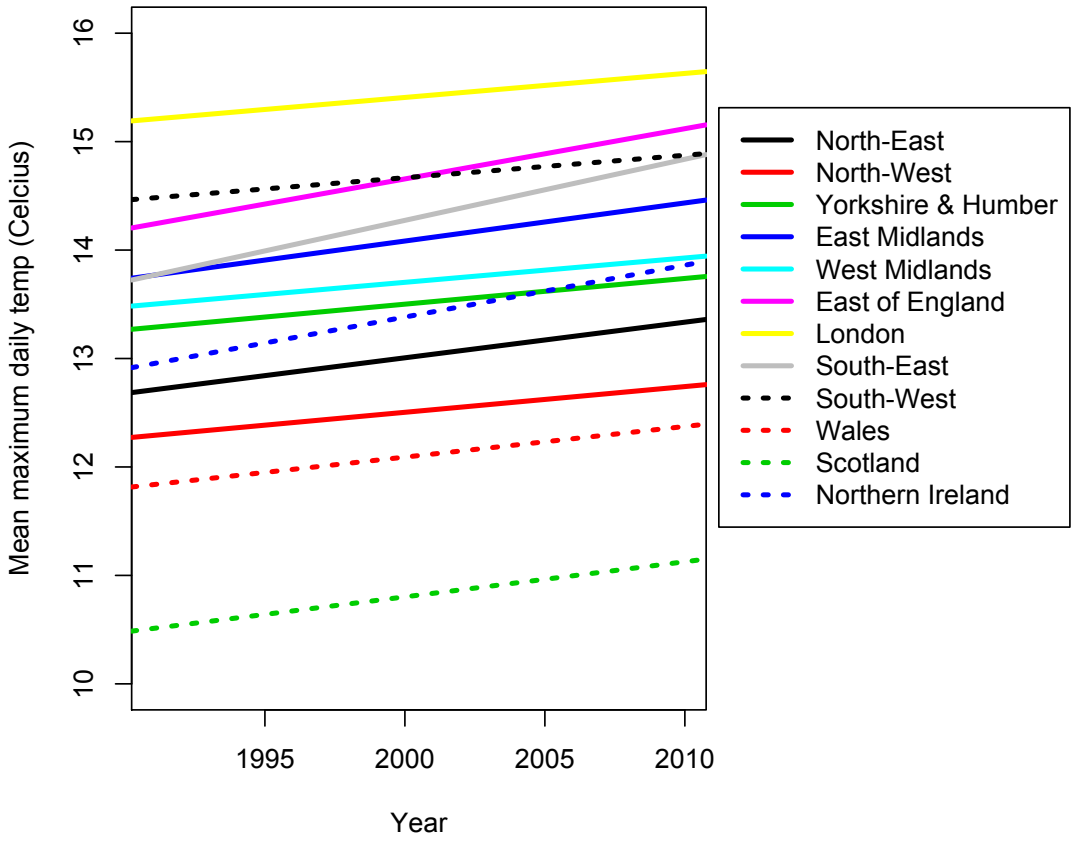



Figure 3.5. Variation in UK temperatures in space and time. Plot shows fitted regression line for each region in study.

Table 3.3. Outputs of twinstim models including model formulae, coefficients and probabilities, and measures of goodness of fit. AIC=Akaike Information Criterion.

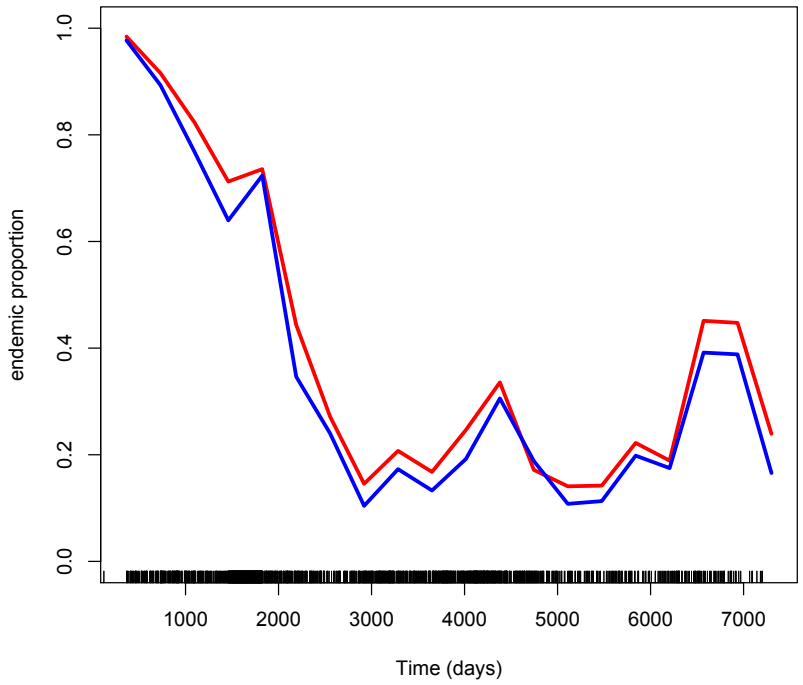
| Model name and parameterisation | Parameter | Estimated coefficient | Standard error | z-value | Probability | Likelihood | AIC |
|---|---|---|---|---|---|---|---|
| Climate - endemic only | Endemic intercept | -22.99114 | 0.41829 | -54.97 | <2e-16 | -20115 | 40234 |
| endemic= ~ 1 + tmax | Average maximum daily temperature | 0.47561 | 0.02886 | 16.48 | <2e-16 | | |
| Population - endemic only | Endemic intercept | -1.66E+01 | 3.15E-02 | -5.26E+02 | <2e-16 | -19871 | 39745 |
| endemic= ~ 1 + popden | Human population density | 6.45E-04 | 1.80E-05 | 3.59E+01 | <2e-16 | | |
| Population plus Climate - endemic only | Endemic intercept | -2.00E+01 | 4.70E-01 | -4.24E+01 | <2e-16 | -19834 | 39764 |
| endemic= ~ 1 + popden + tmax | Human population density | 5.64E-04 | 2.06E-05 | 2.74E+01 | <2e-16 | | |
| | Average maximum daily temperature | 2.42E-01 | 3.31E-02 | 7.30E+00 | 2.82E-13 | | |
| Climate & transmission | Endemic intercept | -20.233271 | 0.700486 | -28.885 | <2.00E-16 | | |
| endemic= ~ 1 + tmax | Average maximum daily temperature | 0.224021 | 0.048711 | 4.599 | 4.25E-06 | | |
| | linear time trend | -0.026421 | 0.008582 | -3.079 | 0.00208 | -17589 | 35190 |
| epidemic= ~ 1 + urbindex + siaf.gaussian + tiaf.constant | Epidemic intercept | -7.48678 | 0.82894 | -9.032 | <2.00E-16 | | |
| | Urban index | -0.46033 | 0.16433 | -2.801 | 0.00509 | | |
| | Spatial interaction function | -0.16148 | 0.01695 | | | | |
| Population & transmission | Endemic intercept | -1.74E+01 | 8.52E-02 | -204.589 | <2e-16 | | |
| endemic= ~ 1 + popden | Human population density | 7.33E-04 | 2.81E-05 | 26.131 | <2e-16 | | |
| | linear time trend | -1.97E-02 | 8.37E-03 | -2.353 | 0.0186 | -17448 | 34909 |
| epidemic= ~ 1 + urbindex + siaf.gaussian + tiaf.constant | Epidemic intercept | -7.30786 | 0.83948 | -8.705 | <2.00E-16 | | |
| | Urban index | -0.45469 | 0.16635 | -2.733 | 0.00627 | | |
| | Spatial interaction function | -0.30707 | 0.01772 | | | | |

## Model Outputs

The model outputs are shown in table 3.3. The population models have higher likelihood and lower AIC scores than the respective climate models. Fitting a simple endemic only model which contains both the population density and temperature predictors significantly improves the model fit compared to the two possible nested models containing just one of these main covariates:

1. Temperature and population density versus population density only model - the more complex model has a lower AIC value and likelihood ratio test confirms data are significantly more likely with both covariates ($D=73$, $df=1$, $p<0.001$).
2. Temperature and population density versus temperature only model - the more complex model has a lower AIC value and likelihood ratio test confirms data are significantly more likely when both covariates are included ($D=562$, $df=1$, $p<0.001$).

There was little variation in the spatial interaction functions between climate and population models (see figure 3.7). Both models predict a rapid decrease in 'infectivity' with distance; ponds will almost never initiate new infections more than 2km away. The proportion of events modeled via the endemic component was also very similar between models of population and climate (see figure 3.6a). The endemic component explains the incidence of the majority of reports in the early years of the emergence but this changes gradually so that by 1997 approximately 75% of new events are modeled via transmission with the other 25% of events arising from outside this system. The proportion of events by region was also visualised and the two hypotheses for spread were compared (see figure 3.6b). The majority of new events are driven by the endemic component except for two regions (London and Staffordshire) in the population model. The climate model has three regions where the epidemic component models the majority of events (the Wirral, London and Staffordshire).



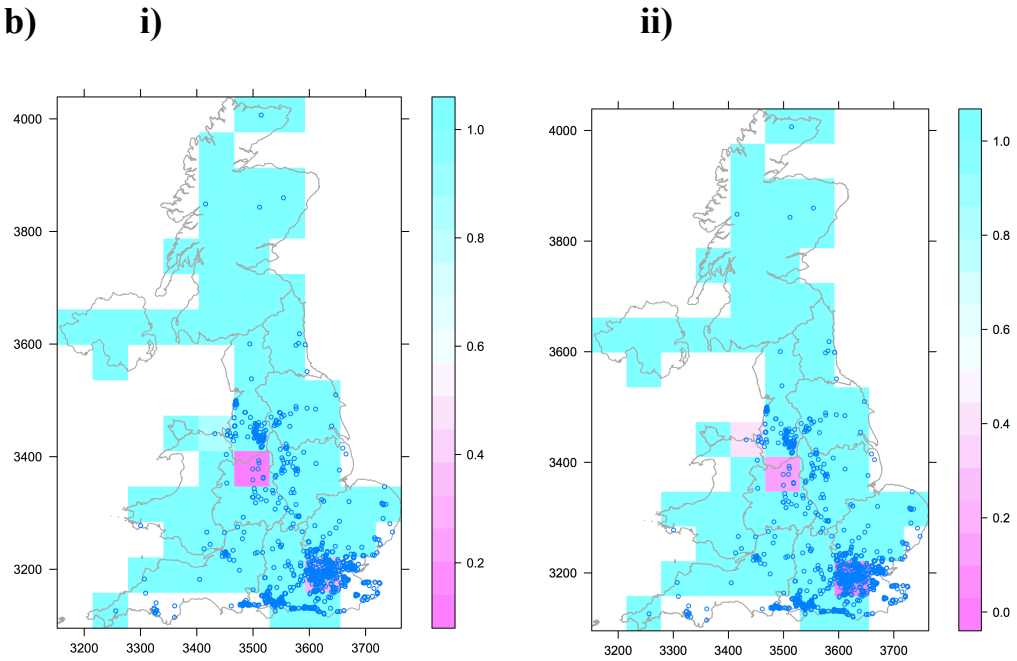




Figure 3.6. Proportion of events explained by the endemic component (events not explained by transmission between ponds) of two-component twinstim models in a) time (Red line shows population model, blue line is the climate model) and b) space (i=population model, ii=climate model).

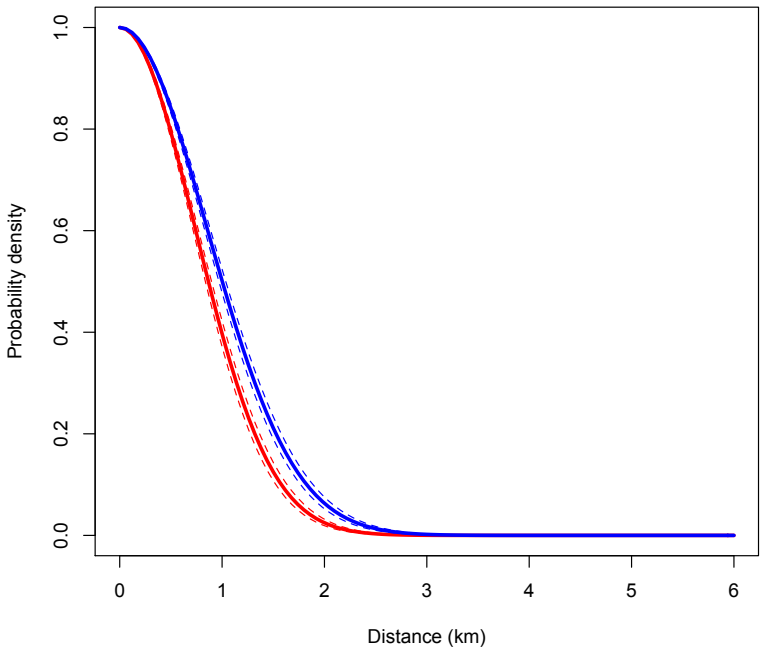



Figure 3.7. Spatial interaction function (siaf) plots showing the decay in 'infectivity' of primary events in space. Red line represents the population model, blue line represents the climate model.

# Discussion

Visualisation of data is a standard first step in spatial epidemiology and previous attempts to visualise spatial patterns of UK ranavirus-consistent mortality through time (for example, http://www.froglife.org/disease/maps.htm) along with genetic evidence supporting a North American introduction (Hyatt et al., 2000) have underpinned the more general assumption that UK ranavirosis is an emerging infectious disease and example of the novel pathogen hypothesis. However, initial visualisation of the data in Figure 3.2 of this study used both ranavirus-consistent mortality events and all other reports of mortality (from hereon, 'negative'). When time series of the two types of mortality event are set side-by-side, it is obvious that there are similar changes in the spatial distributions of both types, suggesting that the perceived pattern of spread could be explained by increased reporting effort alone.

The analysis presented here controlled for reporting effort and number of ponds at risk by standardising the incidence of ranavirus outbreaks by the frequency of the negative

events shown in Figure 3.2. Models were fitted to the data using an endemic component (modelling risk that is independent of the distribution of other infected ponds in time and space) with and without addition of an epidemic component (modelling risk dependent on other infections). The two-component models were a consistently better fit to the data than 'endemic only' models regardless of the composition of the covariates in the endemic component (climate or human population density). This pattern supports the hypothesis that secondary infections, the spread of ranavirus from pond-to-pond, has contributed to the ranavirus spread through the UK.

## Population versus climate

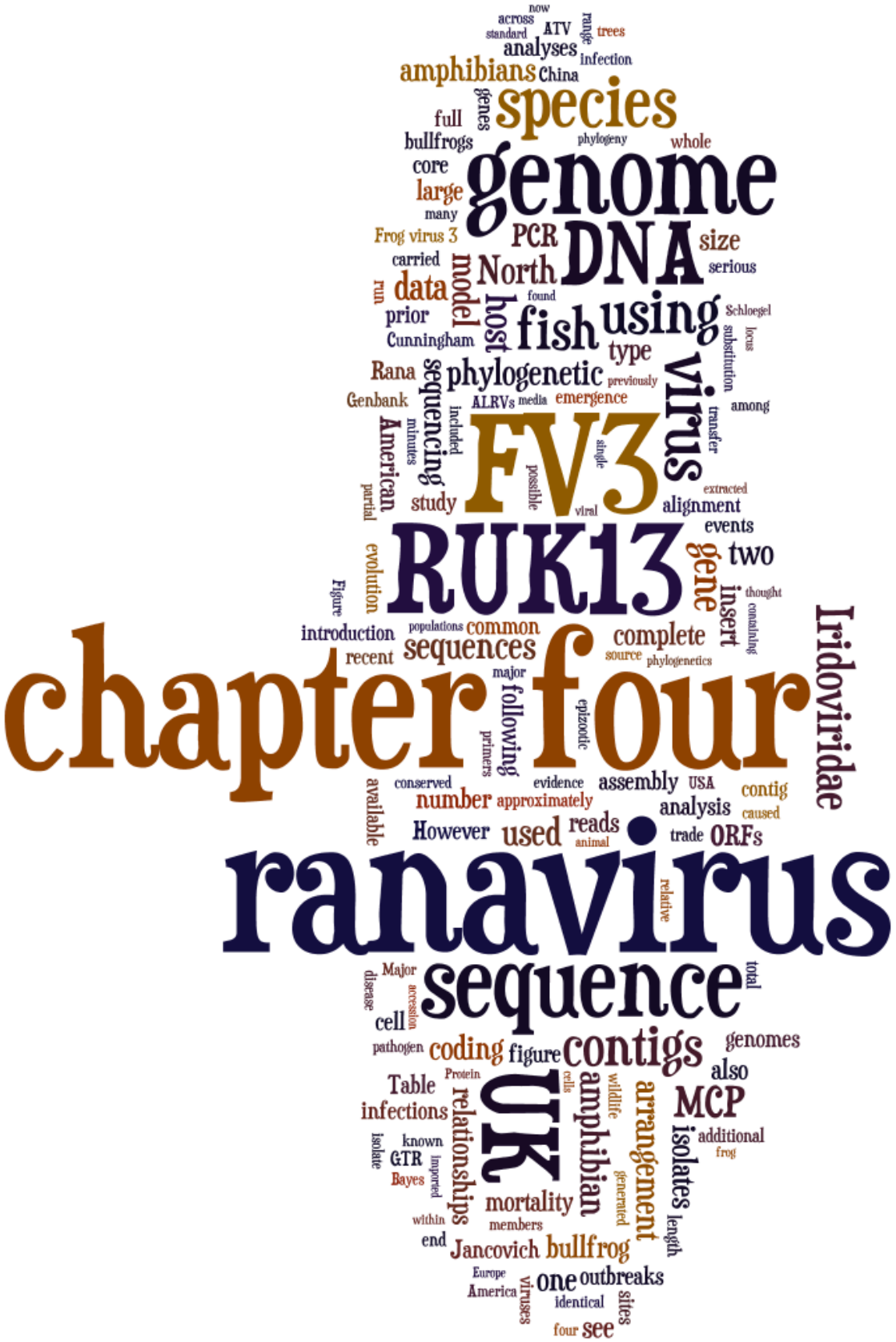
Both models which included human population density were better fits to the data than the respective climate models (they have higher AIC scores, see Table 3.3). An increased incidence of ranavirus-consistent mortality within the most heavily populated regions of the UK (especially Greater London, South-East and North-West England) could be explained if humans introduced more infections by translocation of native species between ponds, or stocking with exotic species and would be consistent with the novel pathogen hypothesis. However an alternative explanation - consistent with the endemic pathogen hypothesis - might involve pollution (e.g. garden pesticides) or some other aspect of human behaviour that has increased in intensity and could have knock-on impacts for amphibian immunity and their susceptibility to ranaviruses. Whilst not confirming translocations as a driver of ranavirus emergence in the UK these findings are consistent with a study in North America, which found a strong correlation between ranavirus incidence and human disturbance and construction (St-Amour et al., 2008) and further strengthen the case for humans being an important driver of this emerging wildlife disease.

When endemic component covariates - population density and temperature - are combined in a simple endemic only model, temperature is retained as a highly significant term (albeit a less powerful predictor than population density) of a model that is a better fit to the data (assessed by likelihood ratio test and AIC) than either variable on their own. Regional temperature explains some of the pattern of spread which population density cannot, but it is difficult to tease apart the individual effects. As with any correlational study it is also possible that an additional, as yet unexplored factor, which is correlated with human population density and/or temperature is the true

driver of emergence. This is a general problem with correlational analyses and emphasises the need for field and experimental data, such as the prevalence of ranavirus in ornamental fish at aquatic centres or exotic amphibians in pet shops. There is only weak evidence of UK warming over the period of this study but the relationship between climate and infectious disease can be more complex than simple long-term trends and it may be necessary to look at small-scale temporal variability in climate when modelling emergence of infectious disease (Raffel et al., 2013).

Pond setting was an important factor in epidemic components of both the population and climate models. The probability of a transmission event at a given time and location increased in urban areas (which account for the majority of mortality events) relative to more rural areas after controlling for reporting effort and the population at risk. This finding contrasts with the expectation that amphibian dispersal is more restricted in urban areas and disagrees with the findings of a population genetics study of urban and rural *R. temporaria* populations (Hitchings and Beebee, 1997). This likely represents a mismatch between the spatial scales used in these analyses and an artifact of the considerable concentration of ranavirus-consistent reports in heavily urbanized areas; 1331 of 1450 events were in heavily urbanized areas. The small percentage of points in rural areas are isolated, more than 30km (the maximum limit of siaf) away from other events and less likely to give birth to secondary infections that would be detected in the survey. The analysis did include compensation for survey-effort, which could in principle accommodate this effect, but the relevant covariates were encoded at regional scale and therefore unable to distinguish between rural and urban areas within one region. The environmental covariates were also recorded at a regional scale, which may also have contributed to a flat likelihood surface and the failure to converge of some models. Both these issues could be addressed if finer resolution data were obtained and the models rerun.

The parts of the UK where transmission between ponds accounts for the majority of outbreaks are geographically concentrated (particularly the two-component climate model, figure 3.6.b.ii), corresponding broadly with the location of ranavirus-consistent reports in the first year of the FMP (1992, figure 3.2a). This is the pattern that would be expected if these areas were the foci of the first infections, whilst in subsequent years the infection was more widely established and arriving via different routes.


chapter four
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# 4

# A serious UK wildlife epizootic is caused by the type species of *Ranavirus*, *Frog virus 3*: the sequence, arrangement and evolution of a UK ranavirus genome

## Abstract

Ranaviruses are virulent amphibian pathogens that have been linked to mass mortality events among common frogs in the UK dating back 25 years. Common frog

populations in the UK that have experienced recurrent mortality events have suffered >80% declines. Trade in amphibians for pets and food is thought to contribute to translocations of ranaviruses and other members of the *Iridoviridae* internationally. A global phylogeny based on a modest amount of sequence data and corroborated by other comparative methods suggested a north American introduction of ranavirus to the UK. Here, I take a whole genome approach to study a UK ranavirus - RUK13 - isolated in East England in 1995, and use phylogenetic and comparative genomics to gain insights into its evolutionary history.

Virus was grown in fish cell culture, purified by ultracentrifugation, and DNA extracted. The resultant mix of fish and virus DNA was sequenced by Roche 454 prior to *de novo* assembly yielding 18 viral contigs with mean coverage depth of 8.34. Genome finishing was attempted using PCR and Sanger sequencing of regions around contig ends after alignment to the genome of a North American isolate (FV3). Phylogenetic analyses were conducted using *Iridoviridae* core genes as well as using the complete coding sequence of the virus Major Capsid Protein (MCP).

The whole genome of RUK13 is presented (Genbank accession KJ538546) and compared to the type species of *Ranavirus*, FV3. Genome arrangement was compared to FV3 by dot-plot revealing two major insertions (>800bp) but otherwise seemingly identical arrangement. The first insert affects the eIF-2α locus where FV3 has a truncated version. The second insertion contains an open reading frame (ORF) with homology to genes in three amphibians and diverse reptiles, raising the possibility of host transfer as a mechanism of recent ranavirus genome evolution.

Overall, the phylogenetic analyses and near identical genome arrangement show that a ranavirus isolated soon after the onset of serious and widespread mortality events in the UK is closely related enough to the type species of *Ranavirus* to be considered an isolate of the same species. These findings also point strongly to an introduction to the UK from North America or from the same source location as North American FV3.

# Introduction

Ranaviruses are globally distributed viral pathogens of amphibians, reptiles and fish and the cause of an emerging infectious disease (EID). Disease outbreaks can affect larvae, metamorphic or adult animals, often causing severe pathology (ulceration and/or systemic haemorrhaging) and mass mortality. Ranavirosis was added to the

World Organisation for Animal Health (OIE) list of notifiable diseases in 2008 (Schloegel et al., 2010) coinciding with and helping to drive increases in reports and awareness of the severe consequences of infection, which include long term host declines (Teacher et al., 2010). Ranaviruses can be divided into two classes on the basis of their phylogenetic relationships: Amphibian-like ranaviruses (ALRVs) and Grouper iridovirus (GIV)-like ranaviruses (Jancovich et al., 2010). A weakness of this terminology is the implication that ALRV host range is restricted to amphibians, which is not the case as ALRVs include viruses for whom fish are the primary host (e.g. *Epizootic hematopoietic necrosis virus*, EHNV). ALRVs can be divided further into ATV-like and FV3-like.

The first isolation of a ranavirus - *Frog virus 3* (FV3) - was from *Rana pipiens* in the USA (Granoff et al., 1966). The virus was isolated in the course of investigations into a tumour but, in spite of this, was not considered to be particularly pathogenic (Chinchar, 2002). A common sign of amphibian Ranavirosis is skin erythema, particularly of the femoral skin, a condition previously termed 'red leg' and once thought to have been caused by a common bacterium, *Aeromonas hydrophila*. Ambystomatid Salamanders had been noted with this and other lesions during recurrent die-offs in Arizona for many years (reviewed in Jancovich et al., 1997). Investigation of such an epizootic in 1995 found a ranavirus - *Ambystoma tigrinum virus* (ATV) - to be the aetiological agent. ATV is now thought to explain disease and die-offs in these salamander populations since at least 1985 but probably date back to the 1970s (Jancovich et al., 1997). FV3 was subsequently implicated in many amphibian disease and mortality events (Green et al., 2002).

In the UK, awareness of and research into ranaviruses followed a similar pattern and time course. Reports of unusual amphibian mortality (mostly among common frogs, *Rana temporaria*) and associated 'red leg' syndrome were received from members of the public from 1985 onwards (Cunningham, 2001). These mortality events were investigated between 1992 and 1995 and a member of the *Iridoviridae* confirmed as the cause of mortality (Cunningham et al., 1996). Some host populations that have suffered recurrent mortality and have undergone severe declines following emergence (Teacher et al., 2010).

Ranavirus infections were known in fish (in Australia (Langdon et al., 1986) and Europe (Ahne et al., 1989)) and amphibians (on the same continents (Fijan et al., 1991; Speare and Smith, 1992)) prior to the publication of the above findings relating to the UK and USA epizootics. Mass mortality events in cultured amphibians in China were also noted from 1995 onwards which have been blamed retrospectively on a ranavirus (Zhang et al., 1999). However most cases have come more recently, with new outbreaks in South America (Fox et al., 2006) and South-East Asia (Une et al., 2009; Xu et al., 2010), and more widespread outbreaks in North America (e.g. Greer et al., 2005). Ranavirus emergence is also accelerating in Europe where infections are now known in Denmark (Ariel et al., 2009), Spain (Balseiro et al., 2010; Balseiro et al., 2009), Italy (Ariel et al., 2010), the Netherlands (Kik et al., 2011), Belgium (Sharifian-Fard et al., 2011) and Germany (Stöhr et al., 2013).

## Pathogen pollution & Emerging Infectious Diseases

One characteristic of an infectious disease that defines it as 'emerging' is an increasing range. Pathogens can expand their range in a number of ways which may be linked to their own biology or that of their existing or surrounding habitat, for example climate change. However, human behaviour can often have an even greater impact on an organism's range through deliberate and accidental translocations. The term "pathogen pollution" was coined for incidences where such human mediated biological invasions involve pathogenic or potentially pathogenic organisms (Daszak, 2000).

Classic examples of pathogen pollution include myxomatosis and rinderpest (Cunningham et al., 2003). A more recent and directly relevant example is provided by the amphibian pathogen, *Batrachochytrium dendrobatidis*, which has been a major driver in amphibian declines following the recent expansion of an extremely virulent generalist lineage (Farrer et al., 2011) present in animals in the amphibian trade (Schloegel et al., 2012).

At present the weight of evidence for an explanation of the mechanism of ranavirus emergence globally favours introduction over endemism. At an early stage in the awareness of ranaviruses as serious wildlife pathogens, North American bullfrogs (*Rana*

*catesbeiana*) (Daszak et al., 1999) and ornamental fish (Hedrick and Mcdowell, 1995) were both put forward as possible vectors of ranaviruses between countries and continents. Amphibians are traded heavily internationally as food and pets with many countries contributing to huge imports into the USA, France and Belgium to satisfy an appetite for frogs' legs (Schloegel et al., 2010). Evidence implicating animal commerce was obtained by Schloegel et al. (2009) who screened nearly 600 imported amphibians at three major US ports of entry and detected ranaviruses in 8.5% of their samples whilst Picco and Collins (2008) had already shown that amphibian trade has contributed to the spread of ATV within the USA. Other ranavirus infections have been associated with introduced amphibians; for example, cultured pig frogs (*Rana grylio*) in China (Zhang et al., 2001) as well as North American bullfrogs in Japan (Une et al., 2009) and Belgium (Sharifian-Fard et al., 2011). Invasive bullfrogs (or sympatric *Xenopus laevis*) are thought to have introduced chytrid to the UK (Cunningham et al., 2005).

The international trade in fish amounts to one billion individuals, comprising more than 5000 species, moving between approximately 100 countries (Whittington and Chong, 2007) and indicates the scale of risk in terms of pathogen pollution. *Lymphocystivirus* was the first genus of the *Iridoviridae* for which members were established as agents of disease in fish (Weissenberg, 1965) and ranavirosis in fish has been observed since the 1980s (reviewed in Ahne et al., 1997). It is not very surprising therefore to find evidence of *Iridoviridae* infection in traded animals. Ten species of fish imported into Korea from other Asian countries each harboured asymptomatic infections with members of the *Iridoviridae* (Jeong et al., 2008). Evidence from microscopy only suggested the presence of members of the *Iridoviridae* in imported, farmed fish species in Israel (Paperna et al., 2001). A survey of retail aquarium shops in Sidney, Australia, for megalocytiviruses found 22% prevalence of infection (Go et al., 2006). On the other hand, a survey for ranaviruses of 753 individual fish (106 diverse species) that died during importation to the EU failed to definitively confirm ranavirus infection in any of the samples (though inconsistencies in the results led the authors to question their own procedures) (Vesely et al., 2011). Overall, *Iridoviridae* infections have been suspected in more than 140 species of fish globally and the virus has usually been shown to be a more FV3-like *Ranavirus* than a *Lymphocystivirus* (Tsai et al., 2005).

In order for fish to serve as a vector of amphibian ranavirus emergence then infections would need to be transmissable between fish and amphibians. Evidence suggests that this is indeed possible: ranaviruses from a frog tadpole and a stickleback (both wild) were apparently identical (Mao et al., 1999), fish can be experimentally infected with an amphibian ranavirus (Moody and Owens, 1994), and amphibians are susceptible to a virus originating from a fish (Bayley et al., 2013). When combined with the increasingly clear picture from ranavirus phylogenetics, the evidence points strongly to the presence of multiple host jumps from fish to amphibians in recent ranavirus evolutionary history (Jancovich et al., 2010).

## Ranavirus genomes

Ranaviruses are large, nucleocytoplasmic, double-stranded DNA (dsDNA) viruses. Genome sizes range from approximately 105 to 140kb which includes 92 to 139 open reading frames (ORFs) and a GC content from 49-55% (Chinchar et al., 2011). Genomes are linear, circularly permuted and terminally redundant (Chinchar, 2002). The number of whole genome sequences available in public databases has been steadily increasing aided by next generation sequencing technologies. There are now ten whole genomes available from the genus *Ranavirus*, and at least one from each of the other genera of the *Iridoviridae*. Comparative studies of ranavirus genomes and efforts to annotate the genomes are ongoing (see Eaton et al., 2007) but the use of a core set of 26 genes conserved across all genera of the *Iridoviridae* is now becoming standard (Mavian et al., 2012; Eaton et al., 2010; Jancovich et al., 2010). Sets of 27 additional ORFs conserved throughout the genus *Ranavirus* and a further 13 ORFs conserved among ALRVs have also been identified (Jancovich et al., 2010). Co-linearity in genome arrangements has commonly been used to understand the evolutionary relationships of ranaviruses, yielding the three ranavirus groups mentioned previously (GIV-like, ATV-like and FV3-like) (Jancovich et al., 2010).

Clearly, the use of a single candidate gene conserved at the family level limits the number of informative sites and these data provide a weaker phylogenetic signal than a whole genome approach. However the Major Capsid Protein (MCP, full coding sequence=1392bp) has previously been recommended as a useful locus for studying *Iridoviridae* relationships (Hyatt et al., 2000; Tidona et al., 1998) and is routinely

targeted in diagnostic tests (following Mao et al., 1996, Mao et al., 1999). As such the MCP has been the most commonly sequenced ranavirus locus and Genbank holds many full-length coding sequences for this gene from ranavirus isolates that are both spatially and temporally diverse.

To date only limited attempts have been made to study phylogenetic relationships of UK ranaviruses. Hyatt et al. (2000) used the MCP approach described above, sequencing a 586bp product which spans the coding and non-coding sequence at the 3' end of the gene. They found two UK ranavirus isolates of common frogs (RUK11 and RUK13) to be very closely related to North American viruses, suggesting a trans-Atlantic introduction to the UK. A more recent attempt has been made to use candidate genes to study diversity and relationships of ranaviruses within the UK (Duffus, 2009). However this study did not examine the relationships of UK ranaviruses to known global diversity among ranaviruses by incorporating available sequences from public databases. In light of the availability of new technologies and increasing availability of ranavirus sequence data, a more comprehensive study of UK ranaviruses is due. I have taken a whole genome approach to better understand the phylogenetic relationships of a UK virus (RUK13) and add to existing knowledge of the arrangement and evolution of ranavirus genomes.

# Methods

## Virus growth

Virus was isolated from a dead and diseased common frog near Ipswich, in Suffolk, England, in 1995 and named RUK13 (Cunningham, 2001). This isolate is known to cause infection and disease experimentally (Cunningham et al., 2007) (see chapter one). Virus stocks were stored in cell culture media at −80°C prior to expansion for sequencing. Aliquots of virus were defrosted at room temperature and passaged three time through fathead minnow (FHM) cell culture (European Collection of Cell Cultures, catalogue number 88102401). FHM cells were grown in sterile, vented, flat-bottomed culture flasks in a standard maintenance media (EMEM plus 10% FBS plus 1% L-glutamine). Cells were maintained at optimal conditions in a $CO_2$ incubator (34°C, 5% $CO_2$) until fully confluent. Virus was added to confluent cell layers using sterile technique and incubated at 27°C. Flasks were monitored daily for signs of cytopathic effect (rounding of cells and plaques in cell layer) and the media

collected once the cell layer was largely cleared (usually four days). Harvested media was spun at 800g to remove cell debris between passages. At the third passage (P3), twenty 75cm$^2$ flasks containing 20ml of maintenance media were each inoculated with 400µl of virus and harvested in 50ml centrifuge tubes after four days as above.

## Virion purification

Concentrating virus particles - Frozen stocks of RUK13 P3 were defrosted overnight at 4°C and spun at 800g for 20 minutes in a benchtop centrifuge to pellet large cell debris. To pellet the virus particles the supernatant was spun at 36000rpm (160000g) for 40minutes using a Beckman Coulter Ultracentrifuge and SW41Ti swinging bucket rotor. The supernatant was discarded and pellets resuspended in RSB buffer (10mM Tris-HCl pH7.5, 10mM NaCl, 1.5mM $MgCl_2$) and stored overnight at 4°C.

Digesting residual DNA from fish cells - $MgCl_2$ and $CaCl_2$ concentrations were adjusted to 10mM and 5mM respectively and 20U of NEB DNAse 1 was added to vials of concentrated virus for each millilitre of virus suspension. This digestion mix was then incubated at 37°C for 90 minutes before adding EDTA to a final concentration of 50mM to block the action of the DNAse.

Recovering virions - the viral suspension was layered atop an 8ml cushion of 20% (w/w) sucrose in RSB buffer and centrifuged for 90 minutes at 30000rpm and 4°C in a pre-cooled SW41Ti rotor. Following centrifugation the upper portion of the sucrose cushion was removed by aspiration and the remaining solution poured off. The pellet containing purified virions was resuspended in 1ml of phosphate-buffered saline and frozen at −20°C prior to DNA extraction.

## DNA preparation: extraction and quality checking

DNA was extracted from purified virions using a Purelink Viral RNA/DNA extraction kit (Invitrogen) according to the supplied protocol. A total volume of 4ml of purified virions in PBS was processed as eight 500µl aliquots. DNA was eluted from each spin column in 10µl volumes giving a total volume of approximately 75µl. RNAs were digested by incubating 40µl of extracted viral nucleic acids with 20µl of

4mg/ml RNAseA for two minutes at room temperature prior to a ten minute incubation at 56°C with 20µl proteinaseK, 140µl PBS and 200µl of Qiagen DNeasy blood and tissue kit buffer AL. DNA was recovered by following the remainder of the Qiagen DNeasy blood and tissue kit Spin column protocol for animal blood or cells with an elution volume of 40µl. A one in ten dilution of this extracted DNA was run on a 2% agarose gel stained with Ethidium Bromide for 105 minutes (75V, 30mA) to assess extraction success and DNA fragmentation and DNA concentration calculated on a Qubit fluorometer.

## Sequencing and assembly

DNA was diluted to 3µg in 100µl of H2O and submitted to Centre for Genomic Research at the University of Liverpool for fragment library preparation and sequencing on one quadrant of a picotitre plate of the Roche 454 GS FLX platform.

*De novo* assembly of sequencing reads was carried out using the Newbler 2.6 assembler (Roche). To avoid bias in the assembly, no reference sequence was provided. The key run settings were as follows: minimum Read Length=20, Minimum Match Length of overlap=40, Minimum Match Identity of overlap=90%, large Genome=False.

## Targeted resequencing

An additional round of sequencing was carried out in an attempt to obtain the necessary DNA sequence to join the contigs from the Newbler assembly. RUK13 contigs and the FV3 genome (Genbank accession, NC_005946) were identical for synteny (see results). Contigs were therefore aligned to FV3 using CLC Genomics Workbench and primers designed using Primer3 (Untergasser et al., 2012) to amplify across gaps in the alignment. Full details of all primers can be found in Appendix B. A new stock of RUK13 template DNA was extracted with a Qiagen DNeasy Blood and Tissue Kit and following the spin column protocol for animal blood and cells.

PCRs were carried out in duplicate wells using a total reaction volume of 25µl (12.5 µl 2X GoTaq HotStart Colourless Mastermix, 1.25µl of 10µM forward primer, 1.25µl of 10µM reverse primer, 5µl of nuclease free water, and 5µl of template DNA). PCR products were divided into two size classes, ‘Large’ (approximately 900bp) and ‘Small’ (approximately 400bp) based on their expected size derived from the alignment of

RUK13 contigs to the FV3 genome. Duplicate reactions containing the standard primers for amplifying an approximately 500bp fragment of the ranavirus Major Capsid Protein (Mao et al., 1996) were included as a positive PCR control for small products. Duplicate reactions containing the standard primers for amplifying an approximately 1000bp fragment of the ranavirus CARD gene (Ridenhour and Storfer, 2008) were included as a positive PCR control for large products. Negative PCR controls containing nuclease free water in place of template DNA were also run along side the above reactions.

Touchdown PCR technique (Korbie and Mattick, 2008) was used to amplify all products. This technique removes the need to optimise individual PCR reactions whilst minimising non-specific amplification. The initial annealing temperature was set several degrees above the calculated annealing temperature for the primers and was then decreased by 0.5°C with each PCR cycle until it gets to 50°C. Touchdown PCR was carried out on Applied Biosystems Geneamp 9700 thermocycler. Reaction settings were as follows: a 10 minute hold at 95°C to activate polymerase, 27 cycles of melting dsDNA at 95°C for 30 seconds, annealing primers at 63°C (decreasing to 50°C) for 30s, elongating new DNA strands at 72°C for 25s (small) or 54s (large), then 15 additional, identical cycles with annealing temp fixed at 50°C, before a five minute hold at 72°C to complete elongation and a hold at 4°C on completion. Samples were stored at 4°C prior to running on a 1% agarose gel stained with ethidium bromide for at least 20 minutes (75V, 30mA) to verify amplification success and confirm fragment size. Successful amplicons were quantified with a NanoDrop 2000 microvolume spectrophotometer, diluted, and submitted to Eurofins for Sanger sequencing of both forward and reverse strands.

## Comparative genome arrangement: dot plots

Genome arrangement of RUK13 was compared, one contig at a time, to the type species of *Ranavirus* (FV3) using Dot Plots constructed in CLC Genomics Workbench.

## Ranavirus phylogenetics

### US22 family protein alignment and phylogeny

US22 family proteins sharing sequence identity to RUK13 open reading frames were retrieved from Genbank using a BLAST search. Amino acid sequences were aligned using the automatic setting of Mafft v7.130b (Katoh and Standley, 2013) and the resulting alignment was edited in Jalview 2.7 (Waterhouse et al., 2009) to remove all gaps. A neighbour-joining tree was constructed from this alignment using BLOSUM62 (also using Jalview 2.7) and rooted on a clade containing only reptilian proteins.

### Phylogenomics

There are 26 core open reading frames (ORFs) conserved across the family *Iridoviridae* (Eaton et al., 2007). VOCs - a web application made up of tools for interrogating a database of viral genomes including members of the *Iridoviridae* - was used to extract individual fasta files representing the 26 core *Iridoviridae* genes from the following genomes; *Frog virus 3* (FV3, AY548484), *Tiger frog virus* (TFV, AF389451), *Ambystoma tigrinum virus*(ATV, AY150217), *Epizootic haematopoietic necrosis virus* (EHNV, FJ433873), *Grouper iridovirus* (GIV, AY666015), *Singapore grouper iridovirus* (SGIV, AY521625), and *Soft-shelled turtle iridovirus* (STIV, NC012637). Genbank contains additional ranavirus whole genomes - *Rana grylio virus* (RGV, JQ654586), *European sheatfish virus* (ESV, NC_017940), *Common midwife toad virus* (CMTV, JQ231222) - that were not available in the VOCs database. All coding sequences for these three species were downloaded from Genbank. The 26 core ORFs for these additional ranaviruses and RUK13 were identified by mapping them to the FV3 genome in CLC Bio genomics workbench and extracting them as fasta files.

Multiple sequence alignments were then calculated using Prank v.100802 for each of the 26 ORFs individually. Alignments were examined in Jalview 2.7 and all gaps removed. Akaike Information Criterion (AIC) and Bayes Information Criterion (BIC) values generated in JModelTest 2.1.1 were used to assess substitution model fit for each of the 26 alignments. Alignments were then joined end to end to create a ‘supergene’ and phylogenetic trees constructed in Mr. Bayes v3.2.1 using a straightforward single partition analysis and after partitioning the sequence by ORFs (i.e. 26 partitions).

The General Time Reversible (GTR) model of substitution was used in the single partition analysis and rates were allowed to vary among sites with a gamma shaped distribution approximated with 4 discrete categories. Stationary nucleotide frequencies were estimated from the data. Two independent analyses were carried out simultaneously using two different random start trees. MCMC was run using Mr. Bayes default settings, i.e. Run length=1000000, sample frequency=500, diagnostic frequency=5000 generations and number of chains=4. The standard deviation of split frequencies and the potential scale reduction factors of the model parameters were used to assess convergence of the MCMC runs as recommended in the program documentation.

I used the model favoured by the AIC whenever there was a disagreement between AIC and BIC. The GTR model with gamma distributed rate variation was the model selected for 24 ORFs, the GTR model with equal rates across sites for one ORF, and the GTR model with a proportion of invariant sites for the remaining one. The substitution model was therefore set by ORF in the 26 partition analysis. Other parameters - including stationary nucleotide frequencies - were unlinked across partitions. MCMC settings were as above for the single partition analysis. Nexus format trees were imported into R and all phylogenetic trees drawn and labelled using the Ape package.

**Candidate gene phylogenetics**

A Blast search for similar sequences to RUK13's full-length MCP sequence revealed a mixture of full and partial (both 5' and 3') sequences. These sequences were downloaded and three multiple sequence alignments - full CDS, 5' partial CDS and 3' partial CDS - generated using Prank. JModelTest was again used to assess best-fit model of nucleotide substitution and tree topology calculated in Mr. Bayes.

# Results

## Sequencer output

The library produced by the Liverpool facilty was highly fragmented, possibly as a result of DNA fragmentation in the submitted sample. A summary of all reads generated by the 454 GS FLX run after adaptor removal is given in Table 4.1. The distribution of read lengths was bi-modal with many short reads.

Table 4.1. Summary statistics for all trimmed reads generated by 454 run.

| | |
|---|---|
| Total filtered reads | 120,599 |
| Total bases | 32,017,515 |
| Mean read length | 265.49 |
| Standard deviation in read length | 120.22 |
| Median read length | 308 |
| Mode read length | 337 |
| 1st quartile | 161 |
| 3rd quartile | 350 |
| Minimum read length | 20 |
| Maximum read length | 826 |
| Range in read lengths | 806 |

## Newbler output

15862 reads - representing 13.15% of the total - were assembled into 2,755 contigs. The total assembly size was 1,040,808bp and mean coverage depth was 5.79. Contigs ranged in size from 100bp to 15,955bp, N50=479bp. Twenty of the contigs were larger than 2000bp and accounted for 131,858 of the total assembly size. Only 18 contigs had significant hits against *Iridoviridae* in Blast homology searches and 14 of these were among the twenty large contigs (276bp to 15955bp in size, see table 4.2). 2.82% of all sequenced bases (2.71% of all reads) mapped to this contig set. Overall mean coverage depth for RUK13 contigs was 8.34.

The majority of the generated sequence data - 97.18% of bases - was therefore from non-viral DNA. The cell line that the virus was cultured in is the most obvious source of contamination of DNA. Homology searches with Blast confirm this; zebrafish

shares significant sequence identity with many of the contigs and the third largest contig seems to be a fairly complete assembly of the fathead minnow mitochondrial genome.

Table 4.2. RUK13 contigs generated by *de novo* assembly of 454 reads – contig length plus total number of reads and coverage depth following read mapping to contigs.

| Contig id | Length (nts) | Coverage depth | Number of reads in assembly |
|---|---|---|---|
| 1 | 15955 | 7.3 | 386 |
| 2 | 15196 | 8 | 398 |
| 4 | 11408 | 7.2 | 275 |
| 5 | 9906 | 8.3 | 271 |
| 6 | 8770 | 8.2 | 226 |
| 7 | 8209 | 9 | 238 |
| 8 | 6742 | 7.6 | 163 |
| 9 | 6402 | 8.4 | 166 |
| 10 | 5128 | 8.1 | 148 |
| 11 | 4546 | 8.2 | 123 |
| 12 | 3712 | 7.7 | 93 |
| 13 | 3361 | 10.2 | 108 |
| 16 | 2764 | 6.5 | 60 |
| 18 | 2368 | 7.5 | 61 |
| 46 | 1362 | 8.4 | 38 |
| 97 | 980 | 5.4 | 19 |
| 859 | 366 | 4.6 | 11 |
| 1267 | 276 | 24.5 | 52 |

**Targeted resequencing: joining contigs**

Sanger sequencing of PCR products produced 24 additional reads covering twelve joins between contigs (i.e. both DNA strands at each of the twelve joins). Integrating these reads with the 18 454 contigs yields four joined contigs.

A PCR was also designed to join contigs 12 and 9 (joined contigs 6 and 11). The reaction produced a faint band of appropriate size when the product was run on an agarose gel, but the sequencing reaction failed to produce a sequence read. This may be due to non-specific amplification.

**Final assembly**

The final four joined contigs (JC6, JC11, JC20, JC28) were combined as a single contig after aligning to FV3 and submitted to Genbank, accession number KJ538546.

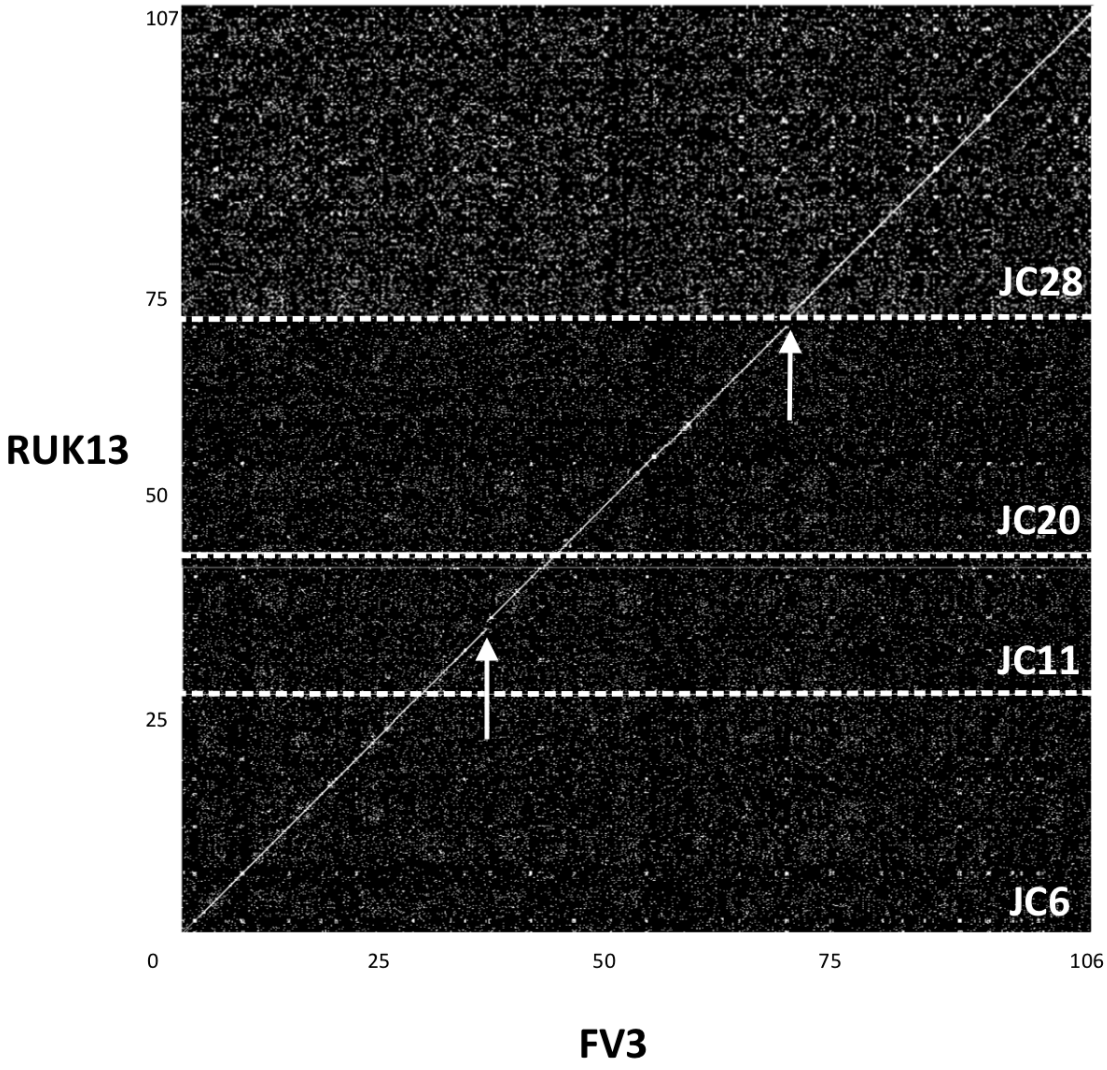



Figure 4.1. RUK13 genome arrangement is highly similar to FV3. Dot plot visualisation of RUK13 contigs with reference to FV3. Arrows show insertions in RUK13 relative to FV3.

## Major insertions in RUK13 compared to FV3

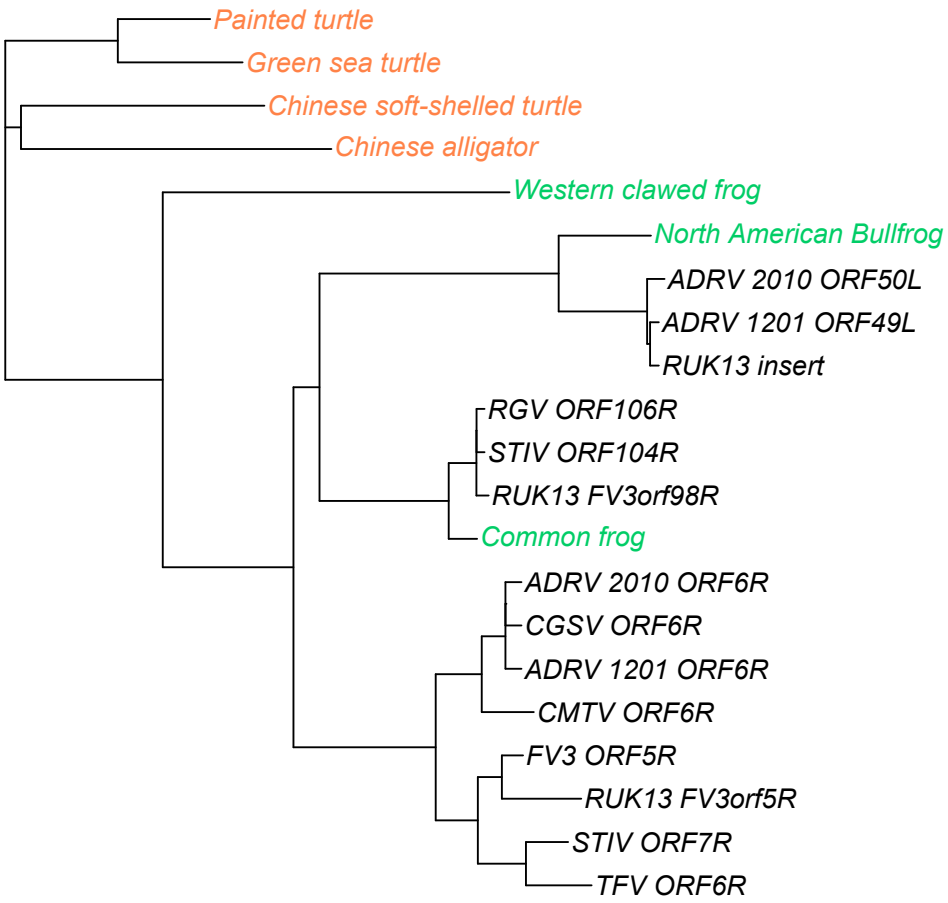
A dot plot comparison of RUK13 contigs and the FV3 genome reveals a 45° line indicating colinearity (see figure 4.1). However there are two major breaks in the line cause by insertions in the RUK13 genome relative to FV3 (marked with arrows in figure 4.1):

1. FV3 ORF26R is a truncated version of the eIF loci. RUK13 has an 800bp insert in this gene relative to FV3. Targeted resequencing of both DNA strands at both ends of the insert support the 454 data.
2. There is a second insert, 1000bp in size, at the end of contig 6 (joined contig 20), which contains an open reading frame. The hypothetical protein contains a conserved motif – US22 – found in vertebrates and DNA viruses. A blast search for homologous sequences (see Table 4.3) revealed highly significant similarities to a putative bullfrog expressed gene, other US22 family proteins in RUK13 and other *Ranavirus* genomes, and an unpublished common frog transcript. Other, more divergent hits included proteins from the Western clawed frog, a number of reptilian species, and avian dsDNA viruses from the families *Poxviridae* and *Herpesviridae*.

The phylogenetic relationships of the *Ranavirus*, amphibian and reptilian proteins are shown in figure 4.2; the *Poxviridae, Herpesviridae,* and other more divergent sequences were not included in this analysis due to the number of gaps in the alignment introduced by inclusion of these taxa. The closest relatives to the putative RUK13 protein contained within the novel insert are proteins from Chinese Giant Salamander viruses, which are joined by the bullfrog transcript in a monophyletic group. There are two other clades of US22 family proteins in ranaviruses and RUK13 contributes orthologs to both. One of these additional clades contains an unpublished common frog transcript that is closely related to the ranavirus sequences whilst the third clade is made up of ranavirus sequences only. There are three amphibian sequences in the tree – Western clawed frog (family: Pipidae) and the common frog and North American bullfrog (both family: Ranidae) – and the topology of the protein tree reflects the overall species tree (see figure 1.1).

Table 4.3. Summary of output from BLAST homology search for proteins matching translated amino acid sequence of RUK13 novel open reading frame.

| Hit | Accession | % identity | % positives | Alignment length | Mismatches | Gap opens | E-value | Bit score |
|---|---|---|---|---|---|---|---|---|
| US22 family-like protein [Andrias davidianus ranavirus] | AGV20580.1 | 99.53 | 100 | 212 | 1 | 0 | 3.00E-154 | 439 |
| hypothetical protein [Andrias davidianus ranavirus] | AHA42318.1 | 99 | 99.5 | 200 | 2 | 0 | 1.00E-143 | 412 |
| FPV250 [Rana catesbeiana] | ACO51661.1 | 85.45 | 90.91 | 165 | 23 | 1 | 3.00E-95 | 288 |
| Expressed transcript, id=233555 [Rana temporaria] | unpublished | 54.26 | 69.68 | 188 | 78 | 3 | 1.00E-62 | 190 |
| unknown [Soft-shelled turtle iridovirus] | ACF42322.1 | 54.4 | 69.78 | 182 | 81 | 1 | 5.00E-56 | 189 |
| herpes virus us22 family like protein | AFG73148.1 | 54.24 | 70.06 | 177 | 79 | 1 | 1.00E-53 | 183 |
| hypothetical protein [Chinese giant salamander ranavirus] | AHA80850.1 | 45.31 | 66.67 | 192 | 99 | 3 | 3.00E-44 | 159 |
| hypothetical protein [Andrias davidianus ranavirus] | AHA42274.1 | 45.31 | 66.67 | 192 | 99 | 3 | 3.00E-44 | 159 |
| FRV [Andrias davidianus ranavirus] | AGV20537.1 | 45.31 | 66.67 | 192 | 99 | 3 | 4.00E-44 | 159 |
| hypothetical protein [Common midwife toad ranavirus] | AFA44910.1 | 52.94 | 75 | 136 | 61 | 1 | 2.00E-42 | 153 |
| orf250-like protein [Frog virus 3] | YP_031583.1 | 50.34 | 71.03 | 145 | 69 | 1 | 2.00E-41 | 151 |
| unknown [Soft-shelled turtle iridovirus] | ACF42226.1 | 51.85 | 71.85 | 135 | 62 | 1 | 2.00E-37 | 141 |
| hypothetical protein [Tiger frog virus] | ABB92274.1 | 51.85 | 70.37 | 135 | 62 | 1 | 5.00E-37 | 139 |
| PREDICTED: uncharacterized protein LOC101932092 isoform X1 [Chrysemys picta bellii] | XP_005283106.1 | 33.89 | 55 | 180 | 111 | 4 | 8.00E-21 | 96.7 |
| PREDICTED: uncharacterized protein 005R-like isoformX1 [Xenopus (Silurana) tropicalis] | XP_002935931.1 | 38.03 | 56.34 | 142 | 79 | 2 | 3.00E-18 | 89 |
| Transmembrane channel-like protein 6, partial [Chelonia mydas] | EMP32440.1 | 32 | 54.67 | 150 | 95 | 2 | 2.00E-16 | 88.6 |
| PREDICTED: uncharacterized protein LOC102455171 [Pelodiscus sinensis] | XP_006110491.1 | 30.77 | 54.49 | 156 | 99 | 3 | 3.00E-16 | 84 |
| PREDICTED: uncharacterized protein 005R-like isoform X1 [Xenopus (Silurana) tropicalis] | XP_004920812.1 | 35.71 | 53.97 | 126 | 72 | 2 | 1.00E-12 | 71.6 |
| PREDICTED: uncharacterized protein LOC102381360 [Alligator sinensis] | XP_006036974.1 | 30.19 | 49.06 | 159 | 104 | 4 | 2.00E-12 | 72.8 |
| PREDICTED: hypothetical protein LOC100680859 [Ornithorhynchus anatinus] | XP_003429952.1 | 27.04 | 47.8 | 159 | 99 | 3 | 8.00E-12 | 73.6 |
| uncharacterized protein LOC422090 [Gallus gallus] | NP_001006423.1 | 30 | 52.31 | 130 | 87 | 1 | 3.00E-11 | 68.2 |
| Uncharacterized protein FPV250, partial [Anas platyrhynchos] | EOB07162.1 | 31.43 | 50 | 140 | 86 | 4 | 1.00E-08 | 60.8 |
| protein SORF2 [Gallid herpesvirus 2] | YP_001034004.1 | 30.36 | 50.89 | 112 | 67 | 2 | 3.00E-07 | 57.8 |
| 15.5 kDa protein [Salmo salar] | ACI68006.1 | 36.59 | 60.98 | 82 | 52 | 0 | 4.00E-07 | 58.2 |
| 15.5 kDa protein [Salmo salar] | ACI67356.1 | 36.59 | 60.98 | 82 | 52 | 0 | 4.00E-07 | 58.2 |
| 15.5 kDa protein [Salmo salar] | ACI67633.1 | 36.59 | 60.98 | 82 | 52 | 0 | 7.00E-07 | 58.2 |
| 15.5 kDa protein [Salmo salar] | ACI68335.1 | 36.59 | 60.98 | 82 | 52 | 0 | 8.00E-07 | 57.8 |
| 15.5 kDa protein [Salmo salar] | NP_001134545.1 | 36.59 | 60.98 | 82 | 52 | 0 | 8.00E-07 | 57.4 |
| SORF2 [Gallid herpesvirus 2] | ACF94950.1 | 29.46 | 50 | 112 | 68 | 2 | 8.00E-06 | 53.5 |
| PREDICTED: uncharacterized protein LOC102778482, partial [Neolamprologus brichardi] | XP_006807842.1 | 25.58 | 48.26 | 172 | 109 | 5 | 2.00E-05 | 53.9 |
| PREDICTED: uncharacterized gene 87 protein-like isoform X1 [Maylandia zebra] | XP_004563698.1 | 29.33 | 48 | 150 | 83 | 6 | 7.00E-05 | 51.2 |
| hypothetical protein FPV250 [Fowlpox virus] | NP_039213.1 | 26.85 | 52.78 | 108 | 66 | 3 | 4.00E-04 | 48.1 |
| PREDICTED: uncharacterized protein LOC102233182 [Xiphophorus maculatus] | XP_005812242.1 | 24.41 | 52.76 | 127 | 87 | 3 | 0.001 | 48.1 |
| protein ORF56 [Anguillid herpesvirus 1] | YP_003358195.1 | 36.59 | 52.44 | 82 | 51 | 1 | 0.002 | 47 |
| PREDICTED: uncharacterized protein LOC102776276 [Neolamprologus brichardi] | XP_006809816.1 | 28.92 | 53.01 | 83 | 57 | 1 | 0.016 | 44.7 |
| SORF2 domain protein [Nile crocodilepox virus] | YP_784211.1 | 26.67 | 52.22 | 90 | 59 | 2 | 0.03 | 44.7 |
| ORF101 [Ranid herpesvirus 1] | YP_656756.1 | 26.72 | 47.41 | 116 | 79 | 4 | 0.2 | 41.2 |
| US420_GAHVG RecName: Full=Uncharacterized 15 | Q05103.1 | 29.41 | 48.24 | 85 | 49 | 2 | 0.34 | 39.7 |
| ORF4 [Fowl adenovirus C] | CCE39396.1 | 26.32 | 53.68 | 95 | 68 | 2 | 0.85 | 38.9 |
| ORF4 [Fowl adenovirus 10] | ABR53703.1 | 26.32 | 53.68 | 95 | 68 | 2 | 0.85 | 38.9 |
| ORF4 [Fowl adenovirus C] | YP_004346955.1 | 25.26 | 53.68 | 95 | 69 | 2 | 2 | 38.1 |
| ORF4 [Fowl adenovirus 4] | ABR53680.1 | 26.32 | 52.63 | 95 | 68 | 2 | 2 | 37.7 |
| non-ribosomal peptide synthetase/polyketide synthase [Corallococcus coralloides DSM 2259] | YP_005370175.1 | 32.86 | 57.14 | 70 | 38 | 3 | 2.5 | 38.9 |
| PREDICTED: vacuolar protein sorting-associated protein 8 homolog [Metaseiulus occidentalis] | XP_003737927.1 | 35.48 | 46.77 | 62 | 36 | 2 | 3.3 | 38.5 |
| hypothetical protein [Aminicenantes bacterium SCGC AAA252-O09] | WP_020261987.1 | 36.36 | 58.18 | 55 | 34 | 1 | 4.4 | 37.7 |
| hypothetical protein [unclassified Aminicenantes] | WP_020260966.1 | 36.36 | 58.18 | 55 | 34 | 1 | 4.4 | 37.7 |
| ORF118 [Ranid herpesvirus 1] | YP_656773.1 | 31.03 | 60.34 | 58 | 39 | 1 | 6.8 | 37.4 |
| putative signal peptide peptidase protein [Neofusicoccum parvum UCRNP2] | EOD44822.1 | 33.33 | 61.67 | 60 | 32 | 2 | 8 | 37 |




**Legend**

| Tip labels | Genbank accession | Ortholog | Taxon name |
|---|---|---|---|
| Chinese alligator | XP_006036974 | n/a | *Alligator sinensis* |
| Chinese soft-shelled turtle | XP_006110491 | n/a | *Pelodiscus sinensis* |
| Green sea turtle | EMP32440 | n/a | *Chelonia mydas* |
| Painted turtle | XP_005283106 | n/a | *Chrysemys picta bellii* |
| Western clawed frog | XP_002935931 | n/a | *Xenopus tropicalis* |
| Common frog | unpublished | n/a | *Rana temporaria* |
| RUK13 FV3orf98R | unpublished | FV3orf98R | *RUK13* |
| STIV ORF104R | ACF42322 | ORF104R | *Soft-shelled turtle iridovirus* |
| RGV ORF106R | AFG73148 | ORF106R | *Rana grylio virus* |
| RUK13 insert | unpublished | insert | *RUK13* |
| ADRV_1201 ORF49L | AGV20580 | ORF49L | *Andrias davidianus ranavirus isolate 1201* |
| ADRV_2010 ORF50L | AHA42318 | ORF50L | *Andrias davidianus ranavirus isolate 2010SX* |
| North American Bullfrog | ACO51661 | n/a | *Lithobates catesbeianus* |
| TFV ORF6R | ABB92274 | ORF6R | *Tiger frog virus* |
| STIV ORF7R | ACF42226 | ORF7R | *Soft-shelled turtle iridovirus* |
| RUK13 FV3orf5R | unpublished | FV3orf5R | *RUK13* |
| FV3 ORF5R | YP_031583 | ORF5R | *Frog virus 3* |
| CMTV ORF6R | AFA44910 | ORF6R | *Common midwife toad ranavirus isolate Mesotriton alpestris/2008/E* |
| ADRV_1201 ORF6R | AGV20537 | ORF6R | *Andrias davidianus ranavirus isolate 1201* |
| CGSV ORF6R | AHA80850 | ORF6R | *Chinese giant salamander iridovirus isolate CGSIV-HN1104* |
| ADRV_2010 ORF6R | AHA42274 | ORF6R | *Andrias davidianus ranavirus isolate 2010SX* |

Figure 4.2. Phylogenetic relationships of US22 family proteins. Neighbour-joining tree constructed using BLOSUM62 and a 147 amino acid Mafft alignment of proteins with significant homology to RUK13 novel protein. The tree is rooted on the clade of reptilian sequences.

## Phylogenetic relationships of RUK13

Table 4.4. 26 core *Iridoviridae* ORFs used in a partitioned 'supergene' analysis of ranavirus phylogenetic relationships. Substitution models: gtr=General Time Reversible (GTR) model, gtr+g=GTR with rates gamma distributed across sites, gtr+i=GTR with a proportion of invariant sites.

| Protein | FV3 ORF | Length | Substitution Model |
|---|---|---|---|
| ATPase-like protein | 15R | 727 | gtr+g |
| D5 family NTPase involved in DNA replication | 22R | 2492 | gtr+g |
| Deoxynucleoside kinase | 85R | 544 | gtr+g |
| DNA-dep RNA pol-II Largest subunit | 8R | 3367 | gtr+g |
| DNA-dep RNA pol-II second largest subunit | 62L | 2970 | gtr+g |
| DNA pol Family B exonuclease | 60R | 2722 | gtr+g |
| Immediate early protein ICP-46 | 91R | 982 | gtr+g |
| Major capsid protein | 90R | 1369 | gtr+g |
| Myristilated membrane protein | 53R | 1306 | gtr+g |
| Myristilated membrane protein | 2L | 641 | gtr+g |
| NIF-NLI interacting factor | 37R | 502 | gtr+i |
| Proliferating cell nuclear antigen | 84R | 656 | gtr+g |
| DNA-dep RNA pol-II Largest subunit | 9L | 2693 | gtr+g |
| Putative replication factor and/or DNA binding-packing | 1R | 698 | gtr+g |
| Putative tyrosin kinase/ lipopolysaccharide modifying enzyme | 27R | 2410 | gtr+g |
| Putative XPPG-RAD2-type nuclease | 95R | 969 | gtr+g |
| Ribonuclease III | 80L | 704 | gtr+g |
| Ribonucleotide reductase small subunit | 67L | 1093 | gtr+g |
| Serine-threonine protein kinase | 19R | 983 | gtr+g |
| Serine-threonine protein kinase | 57R | 981 | gtr+g |
| Erv1/Alr family | 88R | 416 | gtr+g |
| Transcription elongation factor TFIIS | 81R | 225 | gtr+g |
| Helicase family | 21L | 482 | gtr |
| Unknown | 41R | 2978 | gtr+g |
| Hypothetical protein-Clostridium tetani | 94L | 423 | gtr+g |
| Unknown | 12L | 764 | gtr+g |

## Ranavirus phylogenomics

The multiple-sequence alignments from the 26 core *Iridoviridae* genes were concatenated end to end, creating a synthetic supergene 34,097bp in length. The partitions represented by the individual ORFs are shown in Table 4.4 which summarises alignment lengths and substitution models used. Analyses with and without partitions showed complete support for a single topology (see figure 4.3) with only very small differences in branch lengths.

# a)

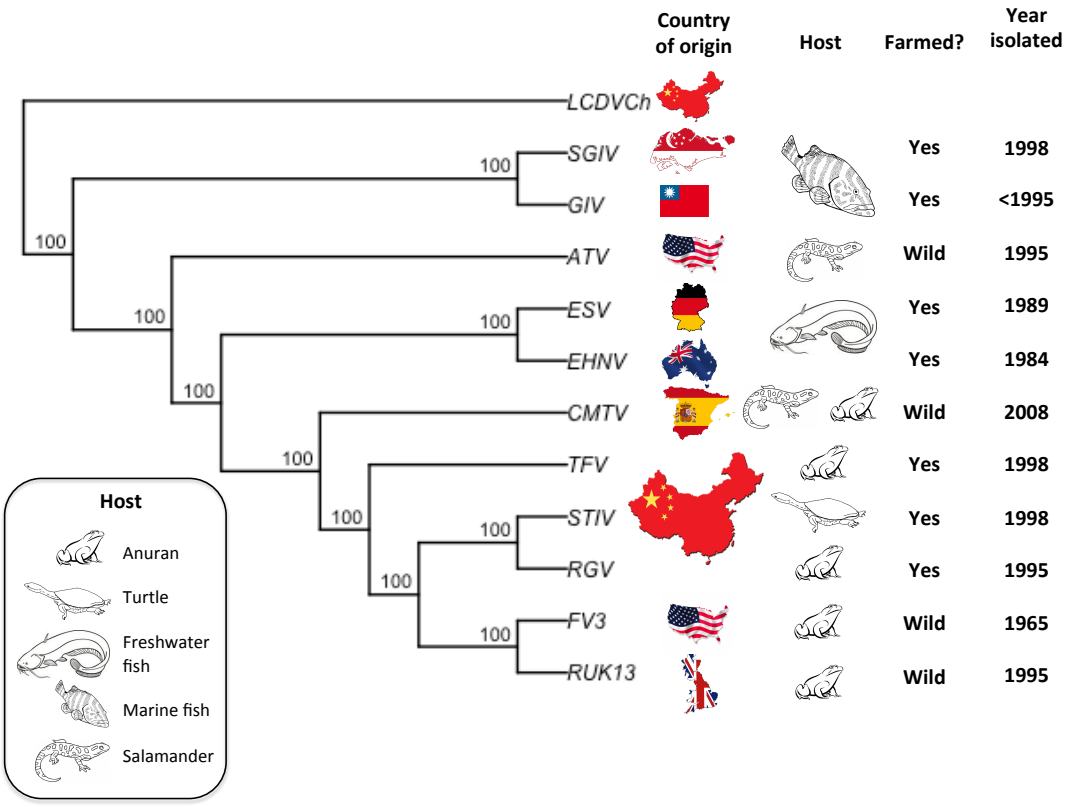



Figure 4.3. RUK13 is the closest relative known to date of ranavirus type species, FV3. Cladogram displaying phylogenetic relationships of ranaviruses based on the 26 core *Iridoviridae* genes with LCDV-China, from the *Lymphocystivirus* genus as an outgroup. Sequences were aligned with Prank and trees constructed using a partitioned model in Mr. Bayes. Node labels are posterior probabilities expressed as percentages.

## RUK13 fine resolution phylogenetics

Three analyses were carried out using Major Capsid Protein sequences, since there is much additional data from other researchers, from samples genotyped only at this locus.

- 38 taxa were included in a 1332bp (after gap removal) alignment of the full MCP coding sequence (see figure 4.4). Isolate names, geographic origins, host species and Genbank accession numbers are provided in Table 4.5.
- 46 taxa were included in a 482bp (after gap removal) alignment of a 5' partial coding sequence of the MCP.

- 60 taxa were included in a 476bp (after gap removal) alignment of a 3' partial coding sequence of the MCP.

Analysis of the complete coding sequence provides a reasonable signal and shows RUK13 in a clade otherwise made up only of FV3 isolates and quite distantly related to other amphibian ranaviruses from Europe: CMTV (from Spain), REV (from Italy), and ZPRV (from Germany) (see figure 4.4). The partial coding sequence analyses did not add anything to the full sequence analysis as it was not possible to resolve polytomies in much of the tree. The MCP gene is highly conserved and - although there were more sequences available - there was a lack of variation between sequences given the shorter length.

Table 4.5. Name, origin, host and Genbank accession number for ranavirus isolates used in the MCP phylogenetic tree.

| Abbreviated name | Isolate | Location | Host | Product | Genbank Accession |
|---|---|---|---|---|---|
| ATV_wg | Ambystoma tigrinum virus | USA | Ambystoma tigrinum | Complete genome | AY150217 |
| BIV_AY187046 | Bohle iridovirus | Australia | Limnodynastes ornatus | Complete CDS | AY187046 |
| BIV_FJ358613 | Bohle iridovirus | Australia | Limnodynastes ornatus | Complete CDS | FJ358613 |
| CGSV_HQ684746 | Chinese giant salamander virus | China | Andrias davidianus | Complete CDS | HQ684746 |
| CGSV_JN615141 | Chinese giant salamander virus | China | Andrias davidianus | Complete CDS | JN615141 |
| CGSV_KC816423 | Chinese giant salamander virus Guizhou | China | Andrias davidianus | Complete CDS | KC816423 |
| CGSV_ADIV | Chinese giant salamander virus strain ADIV | China | Andrias davidianus | Complete CDS | KC465189 |
| CodVirus | Cod iridovirus | Denmark | Gadus morhua | Complete CDS | GU391284 |
| CMTV_wg | Common midwife toad virus | Spain | Mesotriton alpestris | Complete genome | JQ231222 |
| DoctorFishV | Doctor fish virus | Unknown | Doctor fish | Complete CDS | FR677324 |
| EHNV_AY187045 | Epizootic haematopoietic necrosis virus | Australia | Perca fluviatilis | Complete CDS | AY187045 |
| EHNV_wg | Epizootic haematopoietic necrosis virus | Australia | Perca fluviatilis | Complete genome | FJ433873 |
| EuroCatfishV | European catfish virus | Italy | Ameiurus melas | Complete CDS | FJ358608 |
| ESV_FJ358609 | European sheatfish virus | Germany | Silurus glanis | Complete CDS | FJ358609 |
| ESV_wg | European sheatfish virus | Germany | Silurus glanis | Complete genome | JQ724856 |
| FV3_wg | Frog virus 3 | USA | Rana pipiens | Complete genome | AY548484 |
| FV3_DQ897669 | Frog virus 3 | Brazil | Rana catesbeiana | Complete CDS | DQ897669 |
| FV3_FJ459783 | Frog virus 3 | USA | Rana pipiens | Complete CDS | FJ459783 |
| FV3_U36913 | Frog virus 3 | USA | Rana pipiens | Complete CDS | FVU36913 |
| GuppyVirus | Guppy virus 6 isolate F93 | Unknown | Poecilia reticulata | Complete CDS | FR677325 |
| HnebulosusV | Hynobius nebulosus virus | Japan | Hynobius nebulosus | Complete CDS | AB500273 |
| LM_bassV | Largemouth bass virus strain EPC060608-08 | China | Micropterus salmoides | Complete CDS | GU256635 |
| PPIV | Pike-perch iridovirus | Finland | Stizostedion lucioperca | Complete CDS | FJ358610 |
| RCV_JP | Rana catesbeiana virus JP | Japan | Rana catesbeiana | Complete CDS | AB474588 |
| RCV_TW07440 | Rana catesbeiana virus TW07-440 | Taiwan | Rana catesbeiana | Complete CDS | FJ207464 |
| REV_FJ358611 | Rana esculenta virus | Italy | Rana esculenta | Complete CDS | FJ358611 |
| REV_FJ515796 | Rana esculenta virus 50-283-1 | Denmark | Rana esculenta | Complete CDS | FJ515796 |
| RGV_wg | Rana grylio virus | China | Rana grylio | Complete genome | JQ654586 |
| TFV_AY033630 | Rana tigrina ranavirus | China | Rana tigrina | Complete CDS | AY033630 |
| Rv_KRV1 | Ranavirus KRV-1 | South Korea | Rana plancyi chosenica | Complete CDS | HM133594 |
| Ranavirus_maxima | Ranavirus maxima | Denmark | Psetta maxima | Complete CDS | GU391285 |
| SERV | Short-finned eel ranavirus | Italy | Anguilla australis | Complete CDS | FJ358612 |
| STIV_DQ335253 | Soft-shelled turtle iridovirus | China | Trionyx sinensis | Complete CDS | DQ335253 |
| STIV_wg | Soft-shelled turtle iridovirus | China | Trionyx sinensis | Complete genome | EU627010 |
| TFV_wg | Tiger Frog Virus | China | Rana tigrina | Complete genome | AF389451 |
| ZPRV1 | Zuerich Pelophylax collection ranavirus 1 | Germany | Rana esculenta | Partial CDS | KC440841 |
| ZPRV2 | Zuerich Pelophylax collection ranavirus 2 | Unknown | Rana esculenta | Partial CDS | KC440842 |

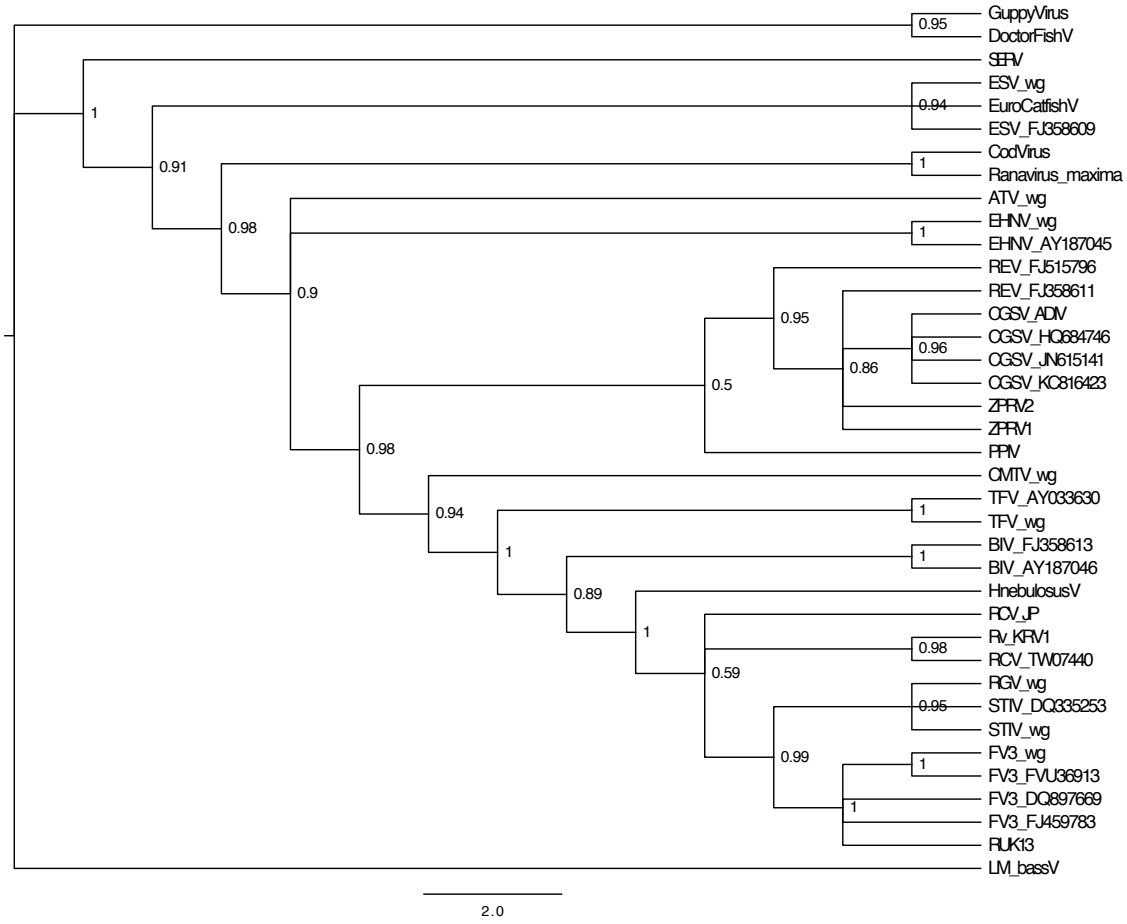



Figure 4.4. Fine resolution Ranavirus phylogeny using the Major Capsid Protein (MCP) complete coding sequence. Sequences were aligned with Prank and trees constructed using a GTR+gamma model of nucleotide substitution in Mr. Bayes. Node labels are posterior probabilities.

# Discussion

454 sequence data covering the whole genome of RUK13 has revealed remarkable similarity to FV3, the type species of Ranavirus. Although I was unable to assemble the genome completely, comparison of the four finalised contigs revealed that they have gene content and gene order almost identical to FV3. Phylogenetic analyses also supported an extremely high degree of similarity between RUK13 and FV3. RUK13 is the closest known relative of FV3 of the virus isolates for which whole genomes are currently available. In the comparison using the subset of the genome for which a greater number of previously typed samples are available for comparison - the MCP analysis - RUK13 remains in a well-supported clade along with four isolates labeled FV3. Three of these FV3 isolates are assumed to be subcultures of the same

original FV3 isolation from *Rana pipiens* (published by Granoff et al., 1966) but the last one is from farmed bullfrog tadpoles in Brazil following import of bullfrogs from North America in 1970 (Mazzoni et al., 2009). Similar analyses to those reported here were carried out on STIV after its genome was sequenced and the authors concluded that FV3 and STIV were isolates of the same virus species (Huang et al., 2009) even though these two viruses infect hosts from different animal classes. The intimate phylogenetic relationship and similarity in genome arrangement between FV3 and RUK13 offers even stronger evidence that they should be considered isolates of the same ranavirus species.

These findings support Hyatt et al. (2000) in demonstrating a close relationship between a UK ranavirus and a North American ranavirus and suggest one or more international translocations. It might seem apparent that the direction of the translocation was easterly - from USA to UK - since FV3 was isolated in 1965 whilst the UK onset of ranavirosis was probably during the 1980s. North American FV3 is also thought of as the source of RGV and subsequently STIV emergence from 1995 onwards in China (Lei et al., 2012). However, it is possible that the translocation occurred in the opposite direction - prior to 1965 - and that RUK13 was either avirulent prior to 1985 or that ranavirosis went unnoticed until then. A further hypothesis involves two introductions (one into the UK, the other into the USA) from the same virus population.

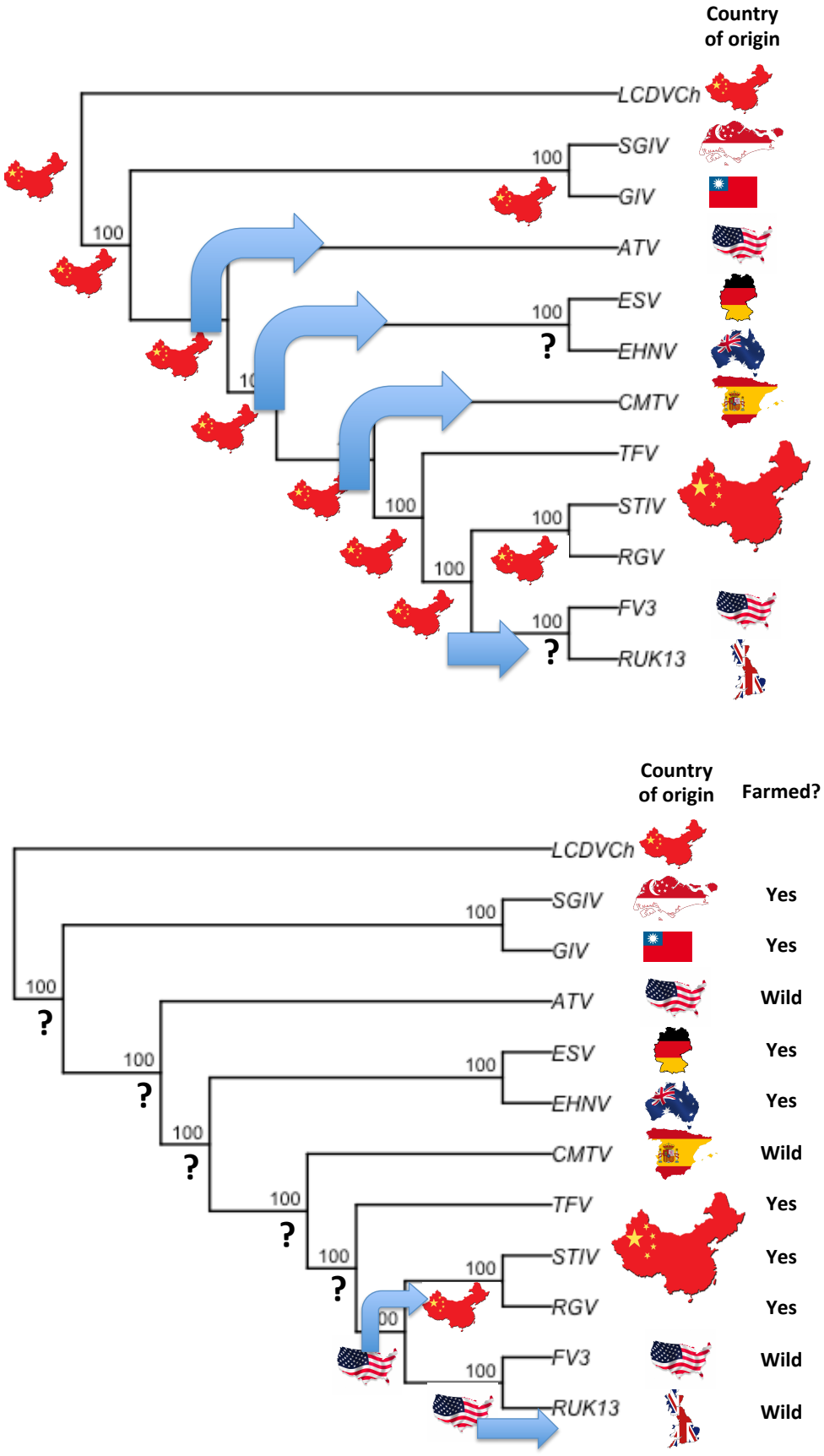



Figure 4.5. Alternate hypothetical patterns of ranavirus migration events a) "Out of China", b) "Into China"

If we limit our thoughts to ranavirus isolates that we have whole genome sequence data for (see figures 4.3a and 4.4), then China and aquaculture become major themes. It is tempting to follow the lead of Jancovich et al. (2010) in attempting to reconstruct the ancestral states on the ranavirus phylogeny but with respect to geography rather than host (see figure 4.5). The simplest reconstruction involves China at almost every ancestral node and four "Out of China" translocations (figure 4.5a). The export of ornamental fish out of China is very likely to have initiated *Megalocytivirus* outbreaks in Japan and Australia (Go et al., 2006). However, this reconstruction does not fully take into account China's enthusiasm for farming fish and amphibians, with aquaculture facilities being responsible for most Chinese ranavirus outbreaks. Since the farms are sometimes stocked by imports (e.g. of *Rana grylio* in the RGV outbreaks), this pattern could support the complete converse "into China" hypothesis (figure 4.5b). Aquaculture is widespread beyond China however and ranavirus outbreaks in Europe and Australia are associated with such practices (Ahne et al., 1989; Langdon et al., 1986) underlining the potential importance of trade and farming internationally. In conclusion, although these data confirm that RUK13 and FV3 have a very recent common ancestor, taken alone they do not identify a source of ranavirus introduction to the UK.

Another interesting feature of ranavirus phylogenetics revealed by this study is the recent huge expansion in the ALRVs shown in figure 4.3b. The fact that a considerable number of emergences affect commercial culturing facilities has two implications: 1) ranavirus outbreaks are being detected regularly rather than evading attention, 2) recent, global trade is further underlined as a strong candidate to explain of the current distribution of ranaviruses.

Both phylogenies (figures 4.3 and 4.4) are also noteworthy for the absence of African ranavirus isolates. The most parsimonious explanation at present is a lack of survey effort in Africa, but given that the virus has emerged recently in Europe and the Americas, surveys of Africa and other regions might uncover evidence of longer-established infections. Such work could parallel the global surveys of chytrid distribution (*Batrachochytrium dendrobatidis,* an amphibian fungal pathogen whose spread has been linked to animal trade); it is surprising that this existing chytrid survey effort has not already produced further reports of ranavirus infection.

Finally, these results show some differences from other studies using the 26 core gene analysis. The ATV-like viruses (ATV, EHNV, ESV) do not form a monophyletic group in this study, disagreeing with Jancovich et al. (2010) and Lei et al. (2012) but agreeing with Eaton et al. (2010) (although they found the positions of ATV and EHNV to be reversed). None of these other studies included ESV in their analyses, which could have improved the current phylogenetic reconstruction; but generally these discrepancies serve to draw attention to the caution needed in interpreting the finer details of phylogenies, even when 26 loci have been used. On the other hand, for the broader features of the phylogeny there is a high degree of agreement between the information garnered from the MCP phylogeny and the 26-core gene analysis. The MCP data performs very well at recovering the same topology as the 26-core gene tree and supports previous assertions that the MCP is useful in determining ranavirus relationships (Hyatt et al., 2000; Tidona et al., 1998).

## Acquisition of host DNA as a mechanism of ranavirus genome evolution?

One of two major inserts in the RUK13 genome (sequences not present in FV3) shared very high sequence identity with a North American bullfrog (*Rana catesbeiana*) transcript, which suggests possible acquisition of host DNA by RUK13 sometime after the divergence of FV3 and RUK13 from a common ancestor. Ranaviruses are classed as nucleocytoplasmic large DNA viruses (NCLDV) on account of their life cycles - which include stages in both the nucleus and the cytoplasm - and their large genome size. Ranavirus genome sizes are at the lower end of this group, which contains *Mimivirus* (genome size, 1.2Mb). Host gene capture is one mechanism by which these viruses have accumulated large genomes (Shackelton and Holmes, 2004) and there is evidence that FV3 has captured host DNA in the past (Filée and Chandler, 2008). A paucity of amphibian sequence data has restricted the usefulness of such analyses until now, but the publication of the *Xenopus tropicalis* genome (Hellsten et al., 2010) and amphibian transcriptome projects (such as Sal-Site; (Putta et al., 2004)) should increase the power to discover other such events.

An alternative explanation of the high sequence similarity between the bullfrog and ranavirus sequences is that the bullfrog host studied harboured a latent infection and that the bullfrog RNA library was in fact a composite library made up of bullfrog and virus transcripts. Homologous proteins containing the same conserved motif (US22) present in the bullfrog and RUK13 sequences are found in other families of dsDNA viruses (*Poxviridae* and *Herpesviridae*) as well as other ranavirus species. As with the bullfrog RNA library, contamination of an unpublished common frog RNA library containing the transcript - included in table 4.3 and the phylogenetic analysis presented in figure 4.2 - could explain the occurrence of a US22 family protein coding sequence. The animals sampled in that study were collected from a pond with a history of ranavirus infection although they were collected at the egg stage and reared in an animal facility under strict hygiene protocols without developing any signs of disease. However, members of the US22 protein family occur frequently in vertebrates (see table 4.3) including the only amphibian with a fully sequenced genome (*X. tropicalis*) and diverse reptiles (figure 4.2). *Ranavirus* US22 sequences are all more similar to these vertebrate sequences than to other known dsDNA virus US22 sequences and, overall, it seems reasonable to infer at least one host transfer during ranavirus evolutionary history.

Homology with a bullfrog sequence invites speculation about the role of this species in the introduction of ranavirus to the UK. Bullfrogs experience ranavirus outbreaks in their native range (Green et al., 2002), they can carry asymptomatic infections (Gray et al., 2007), infected individuals are prevalent within the international trade of this species (Schloegel et al., 2009), invasive populations are infected with ranavirus in Belgium (Sharifian-Fard et al., 2011) and Japan (Une et al., 2009), bullfrogs are suspected of introducing the fungus *B. dendrobatidis* to the UK (Cunningham et al., 2005), larvae of this species were routinely imported to the UK from North America in the 1970s and sold in pet shops and aquatic centres (Langton et al., 2011), and there is a striking overlap between maps of feral bullfrog populations in the UK and the predicted ranavirus distribution in the early years of emergence. Furthermore, a study in the ranavirus-resistant species, *Xenopus laevis*, shows that FV3 can evade immunity in larvae and become quiescent in host macrophages (Robert et al., 2007). Such behaviour in a long-lived species with overwintering larvae would provide an ideal opportunity for lateral transfer of host DNA. The presence of a near identical insert in

the genomes of recently published isolates of *Andrias davidianus ranavirus* (ADRV; Zhou et al., 2013), which is not a closely related species to FV3/RUK13, further complicates efforts to infer the history of this genomic region in RUK13.

The second insertion reveals the existence of both truncated and complete versions of the eIF -2α loci (FV3 ORF26R) within ranavirus genomes. The published FV3 genome contains a 230bp sequence at this locus, encoding a 76 amino acid protein (Tan et al., 2004). The complete genome sequences for STIV and RGV also contain this truncated version of the eIF -2α gene. By contrast, the remaining published ranavirus genomes contain full versions of this gene like RUK13 reported here. The issue is confused by research published prior to the sequencing of the complete FV3 genome. Essbauer et al. (2001) cloned and sequenced eIF -2α genes from five ranaviruses including an isolate they call FV3, citing the original isolation of FV3 (Granoff et al., 1966) - later used for complete genome sequencing - but giving no further details of the source. They report a 780bp sequence (Genbank accession, AF131072) for this FV3 isolate, covering the region that is missing in the complete genome. Other research suggests that the truncated version in the published genome is in fact a genuine coding region (e.g. see Chen et al., 2011) rather than a sequencing or reporting error. However, these inconsistencies do raise concerns over the precision with which virus isolates are described in publications and the possibility of large errors in publicly available data in sequence databases.

I have described the first ranavirus whole genome from the UK. The high degree of similarity with the type *Ranavirus* species is sufficient to justify RUK13 being considered as an isolate of FV3. The data are consistent with the hypothesis that ranavirus emergence results from introduction of a novel pathogen, with North America a likely source and bullfrogs a likely vector – given the link to this species through a putative lateral transfer of DNA from bullfrog to RUK13 following divergence from FV3. Lateral gene transfer from hosts to ranaviruses represents an exciting area of study that warrants further research.


chapter five
host
species
declines
mortality
sites
ranavirus
viruses
Alytes obstetricans
Picos de Europa
amphibian
sequences
three
two
virulent
CMTV
BNV
Spanish
multiple
ranaviruses
animals
infection
isolated
virus
MCP
across
confirmed
Lissotriton boscai
Mesotriton alpestris
Rana temporaria
Ercina
Lloroza
Triturus marmoratus
Balseiro
Moñetas
Ándara
Aliva
Cunningham
Natrix maura
Ranavirus
Iridoviridae
Galicia
Bufo bufo
AAOV
PCR
northern Spain
Highly
die-offs
associated
positive
site
lake
population
among
since
loci
sampled
samples
populations
events
using
also
Spain
CARD
trade
amphibians
diversity
disease
additional
range
one
whole
results
RGV
screened
genes
size
date
found
PdE
ALRVs
Mavian
larvae
time
figure
Figure
infected
groups
whilst
sequence
infections
data
group
five
well
variation
sampling
reservoir
referred
study
identity
Teacher
reports
severe
park
national
therefore
four
mass
introduced
life
dead
another
observed
used
community
introductions
tree
counts
Infections
genomes
history
single
Pontillon
identical
trends
compared
partial
Galician
shown
period
CMTV-like
highly
phylogenetic
diverse
pathogen
Population
Tarentola mauritanica
Bufo spinosus
Pelophylax perezi
Hyla meridionalis
Discoglossus galganoi
Pleurodeles waltl
Salamandra salamandra
Alytes cisternasii
Lissotriton boscai
Triturus marmoratus
Mesotriton alpestris
Rana temporaria
Ranavirus
Iridoviridae
Ascoviridae
Asfarviridae
Phycodnaviridae
Poxviridae
Megavirales
Picos de Europa
Cantabrian Mountains
Galicia
Spain
Lloroza
Cunningham
Natrix maura
Ercina
Balseiro
Moñetas
Ándara
Aliva
Teacher
Mavian
Pontillon
Infections
Bufo bufo
Rana temporaria
Alytes obstetricans
Triturus marmoratus
Lissotriton boscai
Mesotriton alpestris
Pelophylax perezi
Hyla meridionalis
Discoglossus galganoi
Pleurodeles waltl
Salamandra salamandra
Alytes cisternasii
Tarentola mauritanica
Bufo spinosus

# 5

# Highly virulent, introduced viruses in northern Spain are associated with catastrophic declines across multiple host species

## Abstract

Ranaviruses are an important source of mortality in amphibians, which are the world's most threatened vertebrate group, and have been shown to be capable of

causing serious host declines. Ranavirus infections and disease in the UK has been investigated for over 20 years but reports of amphibian mortality events in continental Europe have increased recently. Whilst the possibility of endemic ranaviruses remains, the occurrence of ranavirus infections in animals from the pet and food trade has been shown anecdotally as well as through systematic surveys and suggests that recent introductions are likely.

Amphibian mortality has been observed in the Picos de Europa national park in Spain since 2007 and opportunistic sampling of dead animals has taken place since then, whilst die-offs of thousands of animals at another distant site in northern Spain were sampled in 2010 and 2011. Population monitoring of individual species has also been conducted at 15 sites in the Picos de Europa over the same time period.

With collaborators in Spain, I document mortality across an entire amphibian community (comprising six species) at one site and striking declines in three hosts across three sites where infection with a previously described ranavirus (*Common midwife toad virus*, CMTV) is confirmed. I also propose two new virus species - *Bosca's newt virus* (BNV) and *Ándaran Alytes obstetricans virus* (AAOV) – based on phylogenetic analyses of observed diversity at two partial coding loci. BNV - like CMTV - is a highly virulent pathogen with a broad host range, causing disease and mortality during major die-offs of two newt hosts as well as a spillover snake host. In contrast, AAOV is avirulent to date and was isolated from two asymptomatic animals at a single site.

Lack of monophyly among Spanish virus isolates and the observed patchy distribution suggests at least one pathogen introduction to this region. The capacity of CMTV and BNV to cause mass-mortality and multi-host declines marks out CMTV-like viruses as exceptional pathogens worthy of further research and management efforts.

# Introduction

Amphibians are the most threatened vertebrate class on the planet and whilst numerous diverse causes explain declines, infectious disease is known to have played a key role (Collins and Storfer, 2003). Infections with ranaviruses - large, double-stranded DNA viruses of the family *Iridoviridae* - have been documented on five continents among diverse families (Jancovich et al., 1997; Green et al., 2002; Greer et al., 2005; Fox et al., 2006; Ariel et al., 2009; Balseiro et al., 2009; Cullen and Owens, 2002; Cunningham et al., 1996; Kik et al., 2011; Sharifian-Fard et al., 2011; Une et al., 2009; Xu et al., 2010). Infection is frequently associated with severe pathology and mass mortality but whilst recurrent annual die-offs with ranavirus aetiology have been noted (Teacher et al., 2010; Green et al., 2002), good quality datasets which document declines are usually not available (Miller et al., 2011, but see Teacher et al., 2010). One such dataset comes from the UK where ranavirus infections and mass mortality events in common frogs (*Rana temporaria*) have been recorded and investigated since 1992 (Cunningham et al., 1996). Though occasional infections have been noted in two other common amphibians - *Bufo bufo* (Cunningham, 2001) and *Lissotriton vulgaris* (Duffus and Cunningham, 2010) - confirmed infections and reports of mortality have been focused in *R. temporaria* (Cunningham et al., 1996; Teacher et al., 2010) and median declines of 81% in 18 populations of this species over a 12 year period (Teacher et al., 2010).

Pathogen pollution is thought to be a key driver of emerging infectious diseases (Daszak, 2000) and humans are thought to be significant contributors to both the regional and international spread of the *Iridoviridae*. For example, the use of juvenile salamanders as angling bait in the USA has contributed to *Ambystoma tigrinum virus* (ATV) translocations (Picco and Collins, 2008) whilst, globally, die-offs have often been associated with cultured amphibians (He et al., 2002; Zhang et al., 2001) or introduced species (Une et al., 2009). Schloegel et al. (2009) found an alarming prevalence of both ranavirus and *Batrachochytrium dendrobatidis* among amphibians on sale at three major US ports of entry with similar results available for members of another *Iridoviridae* genus, *Megalocytivirus*, in the ornamental fish trade in Australia (Go et al., 2006) and numerous anecdotal reports of ranavirus infected animals recovered from the exotic pet trade (e.g. see Hyatt et al., 2002; Marschang et al., 2005;

Pasmans et al., 2008). In addition to being prevalent in the amphibian trade, ranaviruses have an extremely broad host range (Miller et al., 2011) which could yield opportunities to become established in new locations even when their natural range is distant and quite different from the new range. Whilst the endemic versus novel pathogen hypotheses have been much explored for another multi-host pathogen of amphibians, *B. dendrobatidis* (e.g. See Farrer et al., 2011; Walker et al., 2010), the same is not true for ranaviruses. Ranaviruses were first isolated almost 50 years ago (Granoff et al., 1966) but have only been associated with amphibian die offs for half of that time. It is often assumed that pathogen pollution with respect to ranaviruses has been coincidental with and responsible for the majority of these mortality events.

In Spain, ranavirus mortality events affecting juvenile alpine newts (*Mesotriton alpestris*) and larval common midwife toads (*Alytes obstetricans*) were described at two nearby sites in the Picos de Europa national park in 2007 and 2008 (Balseiro et al., 2009; Balseiro et al., 2010). The whole genome sequence of *Common midwife toad virus* (CMTV) isolated from the 2008 mortality event was published recently (GenBank Accession No.: FM213466.1). CMTV is confirmed as an Amphibian-like ranavirus (ALRV) with phylogenetic analysis of the 26 core *Iridoviridae* genes and a comparison of the genome arrangements of ALRVs leading the authors to posit an intermediate position for CMTV in the evolution between the EHNV/ATV and FV3 groups of ALRVs (Mavian et al., 2012). Amplicons of a 530bp fragment of the MCP gene from subsequent ranavirus outbreaks in the Netherlands and Belgium were found to be 100% identical to CMTV (Kik et al., 2011; Sharifian-Fard et al., 2011). Beyond these initial reports describing the incidence of mortality events, isolation of a virus and resulting pathology nothing is known about the distribution, diversity and disease dynamics of ranaviruses in Spain.

# Methods

## Study sites and Sampling

Tissue samples (toe-clips and tail-clips from live individuals; whole animals from dead or dying individuals) from a mix of amphibian species and life stages were collected at six sites in the Picos de Europa (PdE) National Park at several time points between 2009 and 2011 and from carcasses of two amphibian (*Lissotriton boscai* and *Triturus marmoratus*) and one reptile (*Natrix maura*) species at the Pontillon reservoir (Galicia) in 2010 and 2011. A full breakdown of the number of samples, tissues sampled, and results of virus screens for each site is given in Table 5.1. The *N. maura* specimen was found dead at the scene during the 2011 amphibian die-off with an inspection of its gut contents confirming that it had been feeding on diseased amphibians. One of the sampled sites - Lloroza - corresponds to the mass mortality event that led to the isolation of CMTV (Balseiro et al., 2010; Mavian et al., 2012). Sampled sites were chosen for ranavirus screening due to the incidence of lesions and mass-mortality among one or more host species present (Ercina, Lloroza, Moñetas, Áliva) or as proposed negative controls given the absence of disease and mass-mortality (Ándara, La Güelga). All sites in the PdE are shown in figure 5.2.

Sites in the PdE are diverse - varying in their elevation, size, amphibian species assemblages, situation, isolation, and degree of human activity. The Galician site is a large reservoir for drinking water, is also used for recreational activities and is home to huge numbers of amphibians. Sampled animals varied by species, life history stage (larvae, metamorphs, juveniles and adults), month and tissue sampled, whether they were alive or dead, site found, and whether they were exhibiting lesions (see examples in figure 5.1). A classification tree was constructed including all screening data from 2011 (in R, using the package ‘tree’, version 1.0-33) to explore how such variation corresponds to results of molecular screening tests.

Figure 5.1. Images of diseased animals observed at sites of mortality. (Clockwise from top-left) *Triturus marmoratus* with severe ulceration; *Lissotriton boscai* with severe eye abnormality; *Alytes obstetricans* adult with severe limb necrosis; *A. obstetricans* larvae with petechial haemorrhaging to left flank.

**Screening and DNA sequencing**

All samples were screened in duplicate using a standard PCR of an approximately 500bp product from the viral MCP gene (Mao et al., 1997) prior to visualisation by electrophoresis on a 1% agarose gel. Positive samples were subjected to an additional PCR reaction to amplify a partial sequence of FV3 ORF64R (ATV ORF40L/41R; CMTV ORF43L), a CARD-like caspase recruitment domain conserved throughout the genus *Ranavirus* (hereon referred to as CARD), (forward primer 5'-CGAGACTGAGAAACCGTA-3', reverse primer 5'-CTCGGCGTCATATTGT-3'). Products were again visualised on a 1% agarose gel. Samples were then submitted to Eurofins for Sanger sequencing of both DNA strands of both PCR products.

**Population Monitoring**

Annual counts of target species within 15 amphibian populations are conducted by park staff in the Picos de Europa (see table 5.2) and collaborators in Spain made this data available for the period of 2007 until 2012. Methodology and the life history stage targeted vary with the size and situation of the water body. Data were analysed for overall trends in population size using TRIM (version 3.0; Van Strien et al., 2000).

Population data are not available at the Pontillon reservoir given the difficulties posed by the size of the site but frequent counts of dead animals have been made (again by collaborators) during mortality events since 2010 (see figure 5.5).

**Association between CMTV infection and host declines**

Fisher's exact tests were used to test for association between CMTV infection and host declines. Tests were performed on data from all monitored hosts (even when there were multiple hosts monitored at a single site) and on data after collapsing sites with multiple hosts monitored to a single data point to account for possible lack of independence within sites.

**Phylogenetic analyses**

Sequences were aligned using PRANK v.100802 (Loytynoja and Goldman, 2005) and edited manually with reference to chromatograms in JalView 2.7 (Waterhouse et al., 2009) to correct for occasional inconsistencies between sequences from complementary DNA strands. JModelTest 2.1.1 was used to select the best fitting model of nucleotide substitution and phylogenies were calculated using the HKY model in Mr. Bayes v3.2.1 (Huelsenbeck and Ronquist, 2001) before drawing and annotating trees in FigTree v1.4.0 (Rambaut and Drummond, 2009). Additional sequences used in alignments and phylogeny construction were downloaded from the NCBI nucleotide database; FV3 (AY548484), TFV (AF389451), ATV (AY150217), EHNV (FJ433873), and STIV (NC012637), RGV (JQ654586), *European sheatfish virus* (ESV, JQ724856).

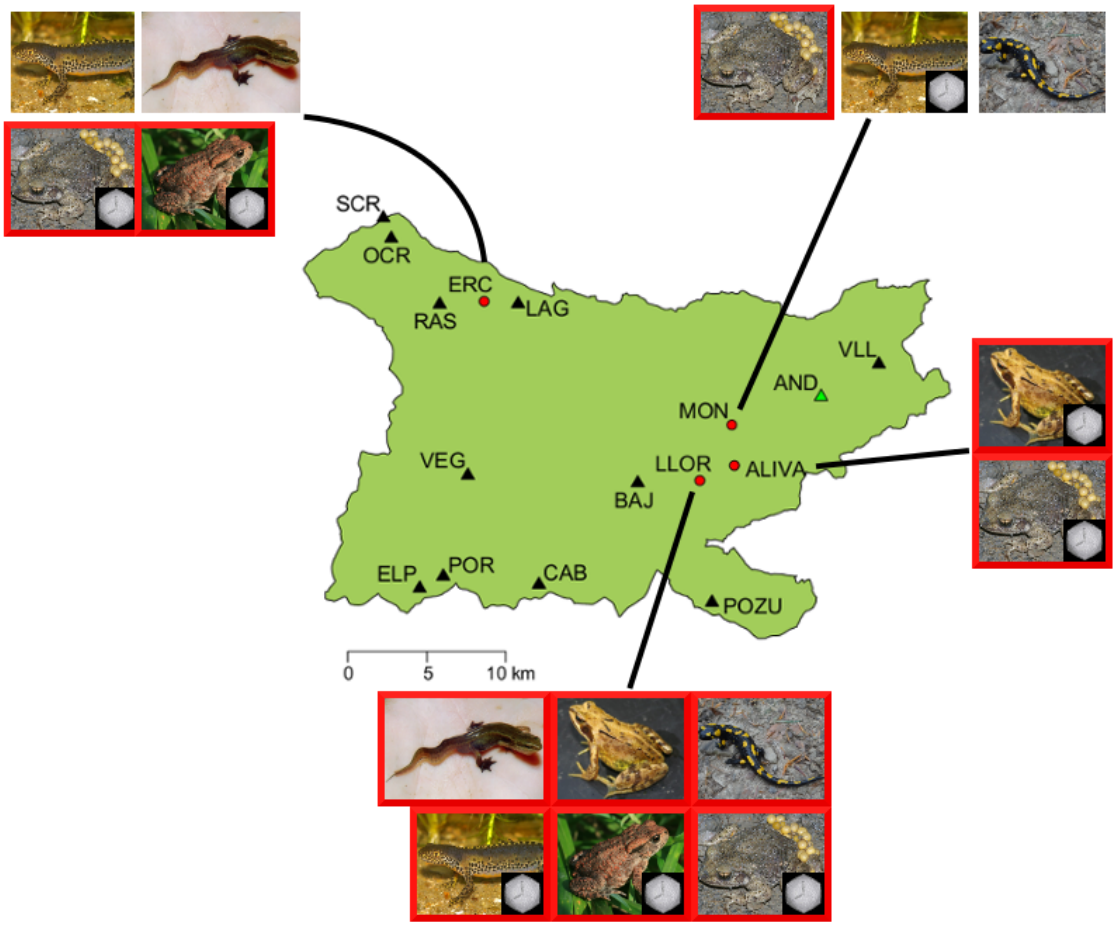



Figure 5.2. Ranavirus infection and mortality among amphibian communities in the Picos de Europa national park. Map shows park boundary and sites where amphibian population monitoring has been ongoing since 2007. Red frame to host images indicates observed mortality. Inclusion of virion symbol attached to host images indicates ranavirus infection confirmed by molecular tests. Red circles mark sites with infection with CMTV-like viruses.

# Results

## Infections: hosts and sites

A sampling summary for samples collected in 2011 - including number of samples by site for each species, proportion of animals which were positive for ranavirus at PCR, tissues and months sampled - is shown in figure 5.3. In total, 34 of 102 samples screened were positive for ranavirus. Ranavirus positive animals of seven different host species were sampled; *M. alpestris* (1 positive from a total of 2 samples screened), *A. obstetricans* (8/67), *R. temporaria* (2/3) and *B. bufo* (1/5) in the Picos de Europa, and *N. maura* (1/1), *T. marmoratus* (14/16), and *L. boscai* (7/8) in Galicia. *A.*

*obstetricans* were infected at three separate sites; Ándara, Ercina and Áliva. Infections were confirmed at five of the seven study sites - Ándara, Ercina, Moñetas, Áliva and the Pontillon reservoir (Galicia) - and at three of these sites (Ercina, Áliva & Pontillon) more than one species was infected. Infections were spread across all life history stages and in live (6), dead (25) and moribund (3) animals.

Table 5.1. Ranavirus screening summary detailing hosts, tissues, timings and results for all sites sampled in the Picos de Europa and additional site in Galicia. Ao=*Alytes obstetricans*; Rt=*Rana temporaria*; Bb=*Bufo bufo*; Tm=*Triturus marmoratus*; Lb=*Lissotriton boscai*; Nm=*Natrix maura*; Ma=*Mesotriton alpestris*.

| Site | Host | Adult | Juvenile | Larva | Tissue | Month | Year | Alive? | Lesion? | Positives | Negatives | Total screened | Prevalence |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ándara lake | Ao | 0 | 1 | 1 | various | various | 2011 | 1 | 0 | 2 | 0 | 2 | 100 |
| Áliva | Ao | 0 | 0 | 2 | Viscera | June | 2011 | 2 | 2 | 2 | 0 | 2 | 100 |
| Áliva | Rt | 0 | 0 | 2 | Viscera | June | 2011 | 2 | 0 | 2 | 0 | 2 | 100 |
| Ercina | Ao | 8 | 11 | 0 | Toe | various | 2011 | 19 | 3 | 4 | 15 | 19 | 21 |
| Ercina | Bb | 5 | 0 | 0 | Liver | April | 2011 | 0 | 0 | 1 | 4 | 5 | 20 |
| Pontillon, Galicia | Tm | 16 | 0 | 0 | various | May | 2010/2011 | 0 | nd | 14 | 2 | 16 | 88 |
| Pontillon, Galicia | Lb | 8 | 0 | 0 | various | May | 2010/2011 | 0 | nd | 7 | 1 | 8 | 88 |
| Pontillon, Galicia | Nm | 0 | 1 | 0 | intestine | May | 2011 | 0 | nd | 1 | 0 | 1 | 100 |
| La Güelga | Ao | 0 | 0 | 20 | Tail | June | 2011 | 20 | 0 | 0 | 20 | 20 | 0 |
| Lloroza | Ao | 24 | 0 | 0 | Toe | June | 2011 | 23 | 1 | 0 | 24 | 24 | 0 |
| Lloroza | Rt | 0 | 0 | 1 | Viscera | June | 2011 | 1 | 1 | 0 | 1 | 1 | 0 |
| Lloroza | Bb | 8 | 0 | 0 | various | various | 2009 | 0 | 0 | 5 | 3 | 8 | 63 |
| Lloroza | Ao | 3 | 0 | 12 | various | various | 2009 | 1 | 9 | 7 | 8 | 15 | 47 |
| Lloroza | Ma | 3 | 0 | 0 | various | various | 2009 | 1 | 0 | 1 | 2 | 3 | 33 |
| Monetas | Ma | 1 | 1 | 0 | various | Sept | 2011 | 0 | nd | 1 | 1 | 2 | 50 |

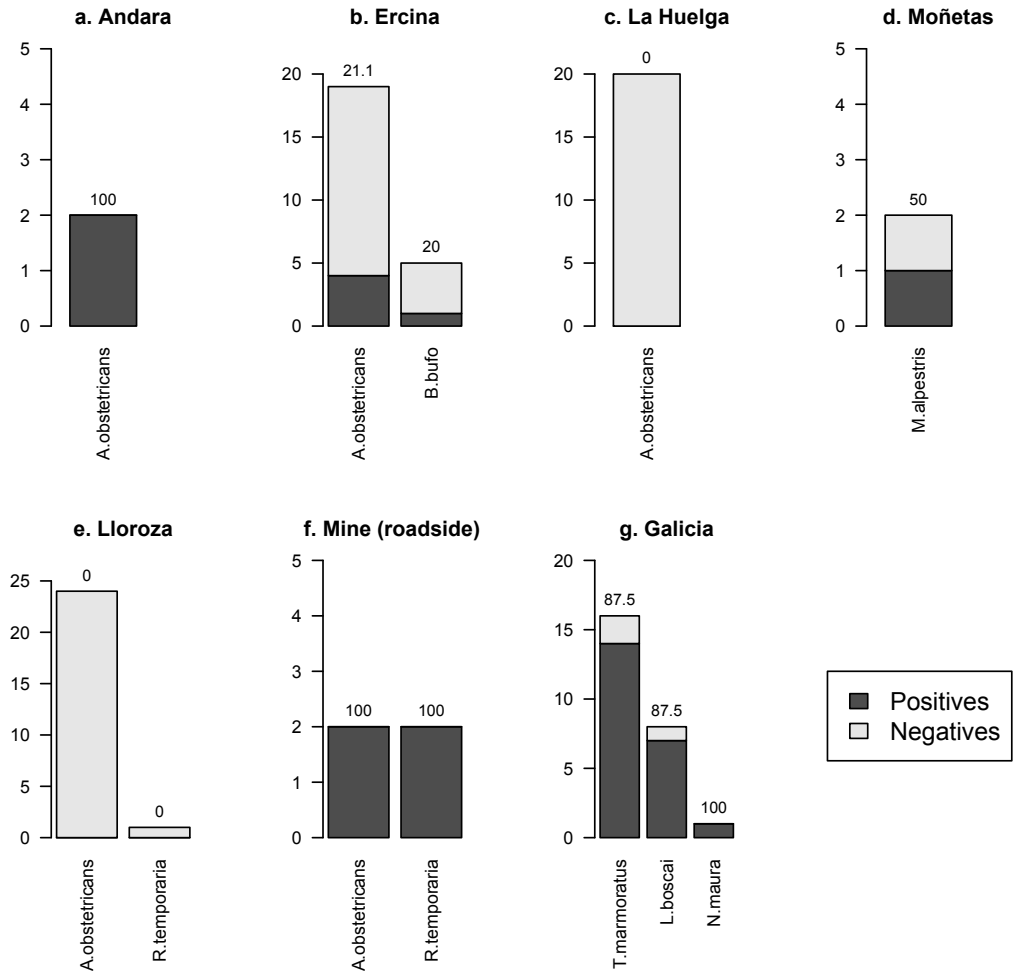



Figure 5.3. Summary of sampling in 2011 showing species and number of samples at each site. Numbers above bars show proportion of positive samples.

A tree model of sampling in 2011 with ranavirus infection status as the response and seven categorical explanatory variables (host species, life history stage, tissue, site, month, host viability and presence of lesions) produced a classification tree with 2/102 misclassifications. At three of the study sites (PdE sites Ercina, La Güelga and Lloroza; accounting for 69/102 samples), month of sampling was important with April and June sampling yielding negative results (one positive *B. bufo* sample was misclassified here) and September yielding positive results. At the other four sites, tissue sampled was important, with liver and viscera producing positive results and differing outcomes for intestines, ovaries and toes depending on the host species sampled.

## Amphibian population trends in the Picos de Europa national park

Population trends in 23 amphibian species were monitored across 15 sites. Ranavirus infection with CMTV-like viruses has been confirmed at three (Ercina, Lloroza, Monetas) of the 15 sites (see table 5.2 and figure 5.6). Five of the six populations monitored at these infected sites are in decline; two have shown steep declines over the study period, with a further three showing moderate declines and one with an uncertain trend. In contrast, at sites where mass-mortality nor ranavirus infection have been detected (15 populations across ten sites), four populations have shown a moderate increase with eight uncertain, two stable, and one showing a moderate decrease. At Soto-Covodonga Road, there has been mass-mortality in one host population along with moderate decline but we do not have infection data at this site. Finally, at Ándara lake, two animals were positive for ranavirus infection with an FV3-like virus (figure 5.6) but no mass-mortality has been observed and the population trend in the target host is uncertain. The data is summarised in table 5.2 and population trends at sites with mortality or ranavirus infection are visualised in figure 5.4.

The incidence of disease, mass-mortality, and infection with CMTV-like viruses is significantly associated with host declines when data is analysed at the level of all monitored hosts (Fisher's exact test, $p=0.0093$) or, more conservatively, when collapsing sites where population monitoring data was available for multiple hosts to a single data point to account for possible lack of independence (Fisher's exact test, $p=0.022$) (raw data included in Appendix C).

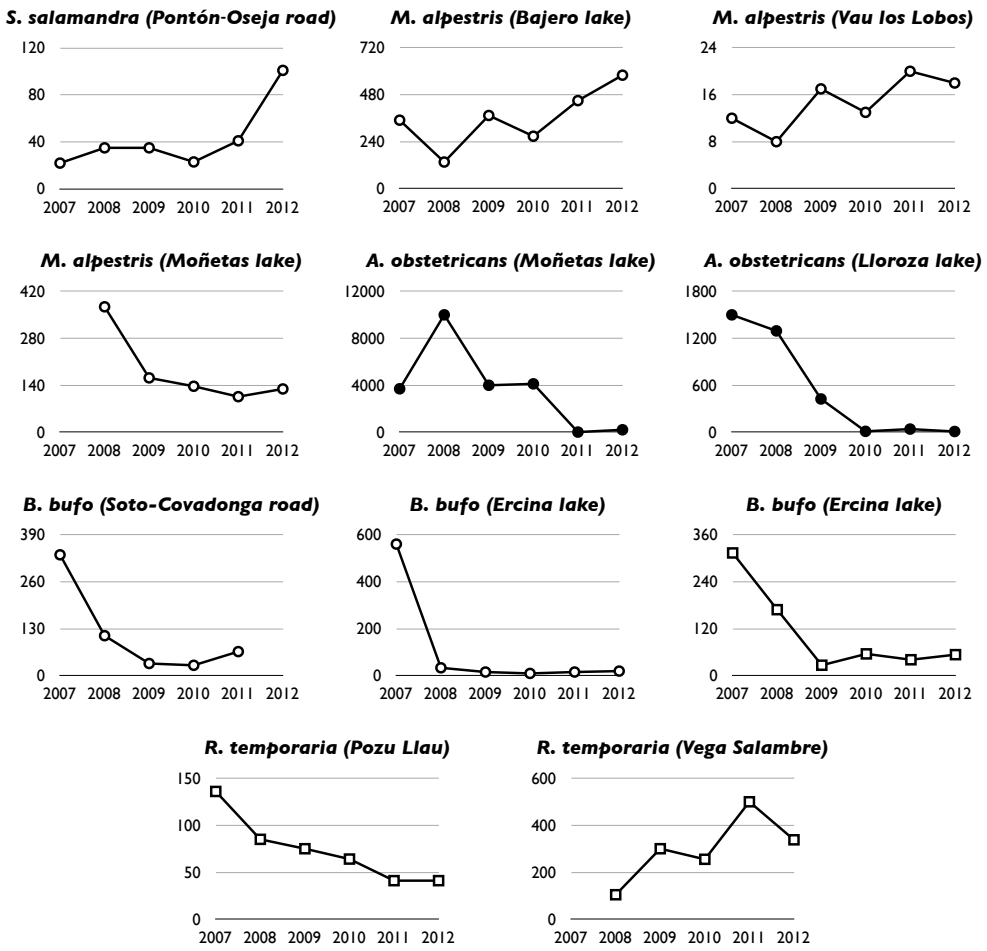



Figure 5.4. Population trends at monitored sites in the Picos de Europa national park. Ranavirus infections are confirmed within the amphibian communities at Monetas, Lloroza, and Ercina Lake. Open circles for counts of adults, closed circles for tadpoles and open squares are for egg-masses.

Table 5.2. Summary of population monitoring and trends since 2007 (calculated in Trim version 3.0 by Spanish collaborator, Jaime Bosch) for multiple hosts and sites in the Picos de Europa national park plus additional site in Galicia.

| | Site details | | | Infection | | Population monitoring | | | |
|---|---|---|---|---|---|---|---|---|---|
| Locality | Abbreviation | Habitat, altitude | Community composition | Recorded mortalities | Virus confirmed | Target species | Target stage | Slope imputed (mean and SE) | Population trend |
| Orandi-Covadonga road | OCR | road, 250 m | *Ss, Bb, Rt* | no | no | *Ss* | adults | -0.0819 (0.1230) | Uncertain |
| Pontón-Oseja road | POR | road, 950 m | *Ss, Bb, Rt* | no | no | *Ss* | adults | 0.0555 (0.0119) | Moderate increase (p<0.01) ** |
| | | | | no | no | *Bb* | adults | -0.0169 (0.0116) | Stable |
| Pozu Llau | POZU | pond, 1856 m | *Ss, Rt, Ao* | no | no | *Ss* | larvae | -0.2003 (0.1535) | Uncertain |
| | | | | | | *Ao* | tadpoles | -0.0597 (0.0346) | Uncertain |
| | | | | | | *Rt* | egg clutches | -0.0553 (0.0048) | Moderate decline (p<0.01) ** |
| Bajero lake | BAJ | small lake, 1875 m | *Ma, Lh, Ao, Rt* | no | no | *Ma* | adults | 0.0280 (0.0130) | Moderate increase (p<0.05) * |
| | | | | no | no | *Ao* | tadpoles | 0.0009 (0.0046) | Stable |
| Vau los Lobos | VLL | cattle tank, 1080 m | *Ss, Ma, Lh, Ao, Rt* | no | no | *Ma* | adults | 0.0459 (0.0152) | Moderate increase (p<0.01) ** |
| Ándara lake | AND | small lake, 1750 m | *Ma, Lh, Ao* | no | AAOV | *Ma* | adults | -0.0277 (0.0523) | Uncertain |
| El Pontón | ELP | pond, 1297 m | *Ma, Lh, Ao* | no | no | *Ma* | adults | -0.0180 (0.0598) | Uncertain |
| La Güelga | LAG | stream pool, 1056 m | *Ss, Ma, Lh, Ao* | no | no | *Ma* | adults | -0.0805 (0.1322) | Uncertain |
| Charcas de Cable | CAB | group of ponds, 1600 m | *Ma, Lh, Rt* | no | no | *Ma* | adults | -0.0111 (0.0320) | Uncertain |
| | | | | | | *Rt* | egg clutches | 0.0107 (0.0205) | Uncertain |
| Moñetas | MON | small lake, 1712 m | *Ss, Ma, Ao* | *Ao* | CMTV | *Ma* | adults | -0.0482 (0.0169) | Moderate decline (p<0.01) ** |
| | | | | | | *Ao* | tadpoles | -0.1736 (0.0494) | Steep decline (p<0.01) ** |
| Lloroza | LLOR | small lake, 1850 m | *Ss, Ma, Lh, Ao, Bb, Rt* | *Ss, Ma, Lh, Ao, Bb, Rt* | CMTV | *Ma* | adults | 0.0102 (0.0203) | Uncertain |
| | | | | | | *Ao* | tadpoles | -0.2471 (0.0535) | Steep decline (p<0.01) ** |
| Soto-Covadonga road | SCR | road, 80 m | *Ss, Bb* | *Bb* | no | *Bb* | adults | -0.0970 (0.0502) | Moderate decline (p<0.05) * |
| Ercina lake | ERC | lake, 1100 m | *Ma, Lh, Ao, Bb* | *Ao, Bb* | CMTV | *Bb* | adults | -0.1288 (0.0644) | Moderate decline (p<0.05) * |
| | | | | | | *Bb* | egg clutches | -0.0734 (0.0341) | Moderate decline (p<0.05) * |
| Rasa Pandecarmen | RAS | pond, 1117 m | *Rt* | no | no | *Rt* | egg clutches | 0.0506 (0.0200) | Moderate increase (p<0.05) * |
| Vega Salambre | VEG | pond, 1318 | *Rt* | no | no | *Rt* | egg clutches | -0.0275 (0.0271) | Uncertain |
| Aliva | ALIVA | roadside pool | Rt, Ao | Rt, Ao | CMTV | nd | nd | nd | nd |
| Pontillon, Galicia | n/a | reservoir | Tm, Lb | Nm, Tm, Lb | BNV | nd | nd | nd | nd |

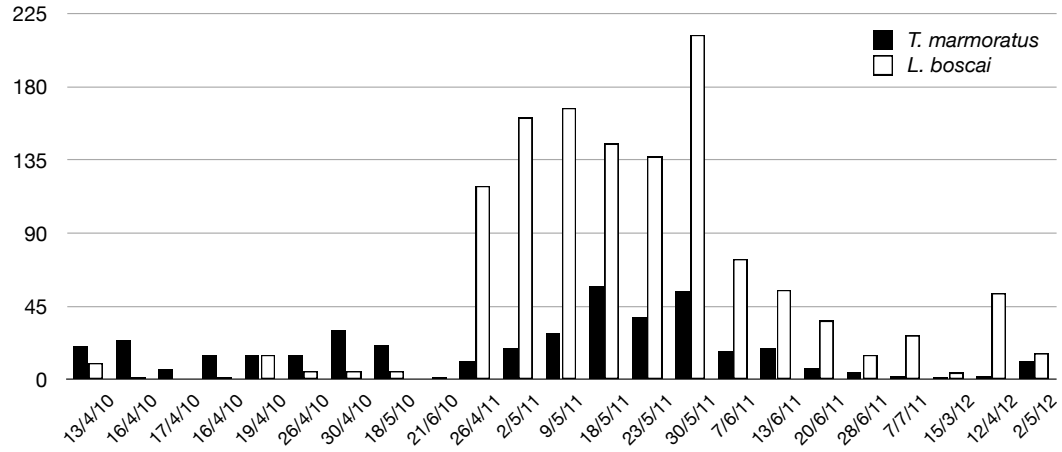



Figure 5.5. Periodic counts of dead animals of two salamander species – *Triturus marmoratus* and *Lissotriton boscai* - at Pontillon reservoir, Galicia between April 2010 and May 2012

**Sequence data and phylogenetic trees**

Sequence data revealed considerable variation in the two genes studied, both among Spanish ranaviruses and when comparing our sequences to overall global ranavirus diversity. Our Spanish sequences fell into three groups with each sharing more than 95.8% identity with MCP sequences from ranaviruses whose whole genomes have been sequenced. CARD sequences were more variable than MCP with a lower minimum identity of 90.8% when compared to the same set of reference sequences (see Methods). The first group were isolated from several hosts (*A. obstetricans*, *M. alpestris*, *R. temporaria*) and sites (Ercina, Áliva, Moñetas) in the Picos de Europa and were 100% identical at both loci to CMTV isolated at Lloroza in 2008 (Balseiro et al., 2010); they are therefore referred to as CMTV-like. The remaining two groups (one from the three species involved in the Galician die-offs, the other from *A. obstetricans* at Ándara lake) were divergent when compared to CMTV and to each other and are referred to as BNV (*Bosca's newt virus*) and AAOV (*Ándaran Alytes obstetricans virus*). CMTV-like, BNV, and AAOV viruses shared at least 97.9% identity between groups at the MCP partial sequence and 95.0% at the partial CARD sequence. The groups formed three distinct lineages (see figure 5.6).

Focusing in on virus sequences from alternate host species, we see no evidence of genetic divergence at a site even when isolated from multiple host species. At the Galician site, BNV sequences from all three sampled hosts (*T. marmoratus*, *L. boscai*, *N.*

*maura*) were 100% identical at both loci for both mortality events (2010 and 2011). In the Picos de Europa, CMTV-like viruses from *R. temporaria* and *A. obstetricans* larvae were also identical at both loci. Identical CMTV-like viruses were sampled from a total of three hosts (*A. obstetricans*, *M. alpestris*, *R. temporaria*) across three sites (Ercina, Áliva, Moñetas).

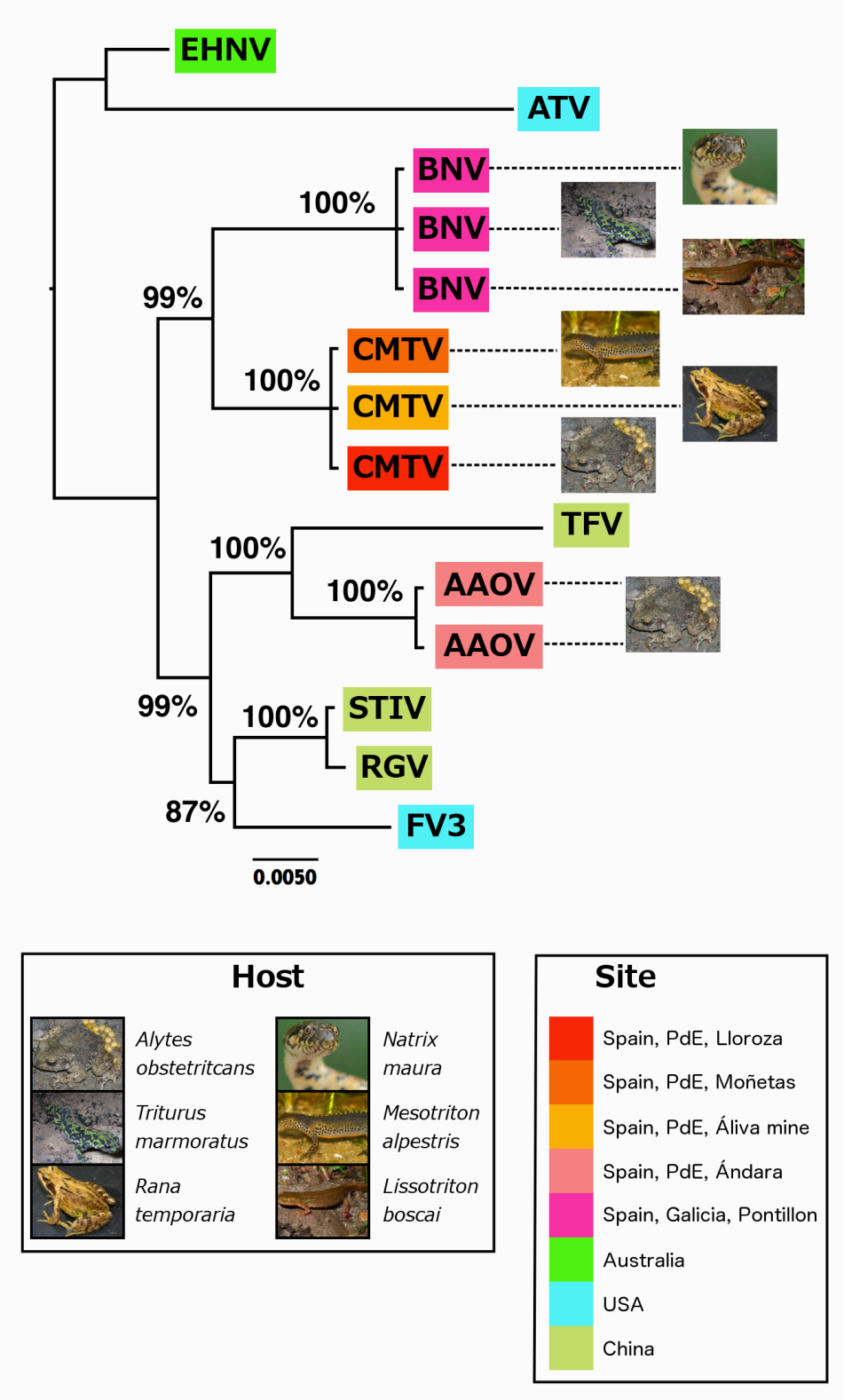



Figure 5.6. Phylogeny summarising known global diversity among Amphibian-like ranaviruses (ALRVs) and including three ranavirus species in northern Spain. The tree was constructed using a partitioned analysis of concatenated MCP and CARD sequences in Mr. Bayes and rooted using ATV/EHNV as an outgroup. Node support values are posterior probabilities expressed as percentages.

# Discussion

Ranavirus infection in the Picos de Europa had previously been confirmed in two host species (*A. obstetricans* and *M. alpestris*) at two nearby sites (LLOR - Lloroza - in figure 5.2) (Balseiro et al., 2010; Balseiro et al., 2009). I uncovered infections in two additional host species (*R. temporaria* and *B. bufo*) at four additional sites across the park (Ercina lake [ERC], Moñetas lake [MON], Áliva mine [ALIVA], and Ándara lake [AND]; see figure 5.2). Wherever ranavirus occurs - with the exception of Ándara lake - we recorded mortality across multiple species combined with infection when sampled. The most striking example comes at Lloroza, home to the largest amphibian assemblage found at any of our study sites (three anurans and three caudates). Mortality has been noted in all six species present with ranavirus infection confirmed by molecular tests in all three species for which we screened multiple specimens; *A. obstetricans*, *M. alpestris* and *B. bufo*.

We have documented catastrophic declines in at least one host at each of these CMTV positive sites for which we have population data. These data are summarised in figure 5.4. *A. obstetricans* populations have collapsed at both Lloroza and Moñetas lakes, with counts of larvae decreasing dramatically. Counts (adults and egg mass counts) of another anuran, *B. bufo*, have undergone a similarly spectacular collapse at Ercina lake. A second species - the caudate *M. alpestris* - has also declined significantly at Moñetas lake over the same period, whilst the population trend at Lloroza in this species is uncertain at present.

Such simultaneous, dramatic declines across host communities following pathogen emergence are both unusual and alarming. Other similar outcomes in animal communities are known and include white nose syndrome in bats (Frick et al., 2010), West Nile virus in birds (LaDeau et al., 2007) and another amphibian disease caused by the fungus, *B. dendrobatidis* (Lips et al., 2006). However, infection of multiple hosts within a community depends on the frequency of transmission opportunities, compatibility between pathogen and host enabling entry and immune evasion and so on. Even variables like the 'pace of life' effect (Johnson et al., 2012) are expected to yield a broad range of host susceptibilities. Furthermore, a community consisting of hosts of differing qualities is considered an important factor which can a yield a 'dilution effect', an effect now shown to occur frequently (Ostfeld and Keesing, 2012). Infection,

mortality and declines due to emerging pathogens are therefore usually expected to focus on a single host species with finch trichomonosis (Lawson et al., 2012) and ranavirosis in the UK (Teacher et al., 2010) recent examples.

Sequence data revealed considerable variation in the two genes studied, both among Spanish ranaviruses and when comparing our sequences to overall global ranavirus diversity. Our Spanish sequences fell into three groups that were distinguishable at both the MCP and CARD loci, with each sharing more than 95.8% identity with MCP sequences from ranaviruses isolated at various locations globally and whose whole genomes have been sequenced. CARD sequences were more variable than MCP with a lower minimum identity of 90.8% when compared to the same set of reference ranavirus genomes.

The first group of Spanish viruses were isolated from several hosts (*A. obstetricans, M. alpestris, R. temporaria*) and sites (Ercina, Aliva, Monetas) in the Picos de Europa and were 100% identical at both loci to CMTV isolated at Lloroza in 2008 (Balseiro et al., 2010; Mavian et al., 2012); these viruses are therefore considered to belong to the same virus species and are referred to as CMTV. A second group of viruses were isolated from carcasses of three species at the Galician study site whilst the final group were isolated from a single host species (*A. obstetricans*) at a single site in the PdE (Ándara lake). These two groups were divergent from CMTV as well as each other, varying at up to 2.1% of sites in the MCP partial sequence and 5.0% of sites in the partial CARD sequence. They are provisionally designated as separate species - BNV (*Bosca's newt virus*) and AAOV (*Ándaran Alytes obstetricans virus*).

Spanish ranaviruses formed three distinct lineages (see figure 5.6). We have used the intermediate position for CMTV in the evolution of ALRVs proposed by Mavian et al. (2012) and based on genome arrangement and phylogenetic analysis of 26 core *Iridoviridae* genes to root our tree on ATV/EHNV. We have shown that CMTV is highly likely to be a cause of mortality and associated catastrophic declines in multiple hosts from diverse taxa. BNV and CMTV form a monophyletic group. BNV represents another highly virulent virus, which is implicated in many hundreds of deaths (see figure 5.5) in two amphibian species (*L. boscai* and *T. marmoratus*) since 2010, as well as spillover into a juvenile squamate, *N. maura*, found dead and infected with BNV

after ingesting amphibians. The final Spanish lineage, AAOV, groups with FV3-like viruses and contrasts with the intermediate viruses (CMTV & BNV) in its seemingly avirulent presentation and restricted host range, with neither mass-mortality nor declines associated with the presence of this virus to date.

A comparison of two Chinese viruses for which whole genomes have recently been sequenced serves to underline the diversity among these Spanish viruses and reinforce the likelihood they represent separate species. RGV and STIV were both isolated from farmed animals in China; RGV from pig frogs in Hubei province in 1998, and STIV from soft-shelled turtles in Guangdong province in 1995 (Chen et al., 1999; Zhang et al., 2001). Almost complete identity between RGV and STIV at the partial MCP (100%) and CARD (99.9%) sequences used in this study disguises overall differences between the two virus genomes, e.g. they share only 84% orthologous open reading frames and are divergent in phylogenetic analyses using the 26 core *Iridoviridae* genes (Lei et al., 2012). It seems likely therefore that the divergence revealed here in CMTV, BNV and AAOV is indicative of substantial differences between viruses at the genomic level.

The patchy incidence of ranavirus infection to date in the PdE, the absence of infection, disease or mortality at other monitored sites in the region and the absence of monophyly of Spanish viruses suggest that ranavirus emergence in this region is mediated via multiple pathogen introductions. Although it is difficult to speculate on the source or means of these introductions, our findings do add significantly to known diversity among ALRVs and are therefore suggestive of further, as yet undiscovered, variation.

We have demonstrated catastrophic declines among multiple species of amphibian as well as simultaneous mortality and decline within host communities. In BNV, we have discovered a sister lineage of CMTV and shown that this monophyletic group of ranaviruses consists of highly virulent pathogens causing infection and mortality across a remarkable host range which includes a snake as well as diverse amphibian families. The presence of an additional, more FV3-like lineage of virus - avirulent to date - also serves as an intriguing contrast to the devastation seen at CMTV/BNV positive sites. Such startling declines and infectivity present considerable management challenges, which are only exacerbated by the likelihood that these diverse viruses represent multiple, recent introductions.


chapter six
genome
UK
Spain
spread
pathogens
hosts
outbreaks
consistent
evidence
infection
DNA
ranaviruses
dsDNA virus
pathogen
disease
experiment
species
model
virulence
viruses
infections
virus
mortality
human
infectious disease
host
evolution
emergence
ranavirus
range
sampling
likely
genetics
populations
recent
ponds
Ranavirus
introduction
approach
may
known
well
novel
suggests
potential
described
ability
isolates
amphibian
sequencing
domestic
multiple
data
years
animals
two
transmission
broad
pattern
chapter
pollution
insights
garden
also
whole
observed
impacts
transfer
history
whilst
higher
used
models
therefore
introduced
identification

# 6

# Summary and prospects

Ranavirus infections in the UK have caused the deaths of thousands of common frogs annually for more than twenty years and research efforts have yielded insights into pathology and infectivity as well as impacts on host populations and their responses. This thesis extends understanding of Europe's most devastating cases of ranavirus emergence, located in the UK and northern Spain, where the consequences of infection for susceptible species are grave.

A striking difference between the two systems is the host range of the respective pathogens. In the UK, infection is highly host-specific and spillover is rare and unlikely to lead to decline in the spillover host (Duffus, 2009). In contrast, mass mortality events can affect entire amphibian communities in Spain - involving up to six

diverse host species. UK and Spanish viruses are from distant lineages and the UK is unlikely to have been the source of ranavirus emergence in continental Europe. Instead we appear to be confronting two recent, distinct and ongoing ranavirus emergence events, both of which threaten amphibian biodiversity. There are therefore clear conservation implications of both emergences, but the research implications reach beyond this emerging infectious disease in amphibians.

Most human pathogens are zoonotic, jumping hosts from wildlife or domestic animals (Woolhouse et al., 2001). About 40 new viral EIDs have been discovered in humans over the last fifty years as a result of host range expansion - including SARS, MERS, Ebola and avian flu. Viruses are well suited to exploit novel hosts due to their short generation time, high fecundity and mutation rate, and frequently an extraordinary ability to evade host immunity (Alcami and Koszinowski, 2000; Cleaveland et al., 2001).

Advances in sequencing technologies have brought about rapid increases in knowledge regarding the content and evolution of virus genomes in the past decade (Koonin and Dolja, 2006). Duplication, horizontal transfer, recombination and mutation are all processes known to generate variation (Filée and Chandler, 2008; Kirzinger and Stavrinides, 2012). Unfortunately, we still understand little of the genetic determinants of host range (Altizer et al., 2003), which in turn hampers our ability to respond effectively to emerging disease threats. As such, the range of virulence and host range observed in ranaviruses, their resultant conservation impacts, and the size and ability to manipulate their genomes makes them ideal potential models to yield much needed insights into host jumps and disease emergence.

## Ranavirus epidemiology: tearing open the box

Prior to 2009 ranavirus diagnostics relied almost exclusively on sampling visceral organs from carcasses. In the absence of mortality, surveillance using this existing sampling protocol required live animals to be removed from the wild and therefore did not generally occur. Estimates of ranavirus incidence were necessarily limited to regions and species exhibiting gross signs of disease or mass mortality. Even when it was possible to sample carcasses, information about sub-clinical infections was still lacking, and the estimation of fundamental epidemiological

parameters (e.g. infection prevalence) remained challenging. The exposure experiment in Chapter 2 provides a remedy to this problem.

The controlled assessment of swabs and toe-clipping in comparison to traditional sampling protocols supports the findings of other recent studies (Gray et al., 2012; Picco et al., 2007; St-Amour and Lesbarreres, 2007), in establishing that non-lethal and non-invasive sampling protocols can be useful in screening for ranavirus infection, albeit with the caveat that no one sampling protocol is 100% sensitive in all cases. The same experiment also provides evidence for a lethal threshold with regards to pathogen burden in hosts and suggests that previously described disease syndromes may actually be stages in disease progression. Finally, a set of pathological changes in hosts that were consistent indicators of infection – ulceration and petechial haemorrhaging - was established.

## The link to humans: two smoking guns

The work in Chapter 2 provided a systematic identification of typical ranavirus lesions with statistical support. This served to validate the filtering methodology used to extract ranavirus-consistent events from a citizen-science database of reports of unusual frog mortality. These data were then used in spatio-temporal models of the spread of ranavirus-consistent outbreaks in chapter 3; an approach which complemented the whole genome approach of chapter 4 where I describe the first UK ranavirus genome.

The combination of results from chapters 3 and 4 establishes a much clearer picture of ranavirus emergence in the UK. The strong evidence presented in chapter 4 supports UK ranaviruses as novel, introduced pathogens and adds to previous work documenting ranavirus translocations (e.g. (Jancovich et al., 2005) whilst representing the most compelling case to date of an international movement initiating an epizootic in native species. I have shown that this UK isolate is a variant of the type *Ranavirus* species (FV3) and confirmed that *Ranavirus* has been introduced to the UK at least once. North American bullfrogs are heavily implicated in this introduction due to the presence of a novel insert in the RUK13 genome relative to FV3 (see below), which could have been acquired from a bullfrog host. Bullfrogs were routinely imported to the UK and sold in pet shops and aquatic centres in the 1970s and have established several breeding

populations in the South-East of England in recent decades; a timing that is coincidental with the earliest confirmed cases of ranavirus infection. Ranavirus infection in traded animals seems to be widespread (Picco and Collins, 2008; Schloegel et al., 2009) with some hosts capable of harbouring latent infections, and makes pathogen pollution (Daszak et al., 2000) a likely broad driver of emergence. Rinderpest, rabies, canine distemprer virus, phocine distemper virus are all examples of serious outbreaks of disease in animal hosts initiated via pathogen pollution (Cunningham et al., 2003) whilst in plants ash dieback may have spread in Europe via the international trade in young, infected trees between nurseries.

The pattern of spread within the UK is best described by epidemiological models that include transmission of infections between nearby ponds, suggesting that ranavirus infection is indeed spreading by this route. However, human population density is also shown to be a key factor in ranavirus emergence - outbreaks appear at a higher rate where human population density is higher. The cause of this correlation cannot be unequivocally established from the outbreak data alone: for example ponds near dense human populations may be more likely to receive translocated frog spawn (consistent with the novel pathogen hypothesis); alternatively the pattern could be due to direct human influences on the environment or landscape, such as increased levels of pollution making the frogs more susceptible to infection (consistent with the endemic pathogen hypothesis). St-Amour et al. (St-Amour et al., 2008) used a quite different approach when studying incidence of ranavirus infection in Canada, but also found a correlation between infection and human activity. Reports of ranavirosis in UK amphibians have focused on domestic garden ponds where human activities have aided the emergence of another infectious disease of garden wildlife in recent years. The use of bird feeders without regular disinfection regimes or rotation of their position in gardens has facilitated the spread of avian poxvirus infections (Lawson et al., 2012).

In the context of the phylogenetic and whole genome analyses of chapter 4, which provide strong evidence for an introduction, UK ranavirus emergence can now be viewed as a novel invasive pathogen with confidence.

## The bigger picture: a glimpse at ranavirus evolution, fundamental niche and life outside the UK

Beyond this central question about emergence chapters 3 and 4 contain other exciting findings. Horizontal transfer of host DNA is known to happen frequently in some dsDNA viruses – e.g. poxviruses (Bratke and McLysaght, 2008; Hughes et al., 2010) – and is likely to have occured in ranaviruses (Filee, 2009). I have uncovered a possible case of horizontal transfer from an amphibian host in RUK13 following whole genome sequencing and *de novo* genome assembly. The insertion of an approximately 1000bp segment of DNA contains an ORF with a conserved (US22) domain, which is known in vertebrates as well as other DNA viruses and is predicted to aid in immune evasion through interaction with specific host proteins. The identification of the genomic insert with very high identity to a bullfrog transcript suggests a recent gain of genetic material from a host and, as well as providing a clue as to the virus' colonisation history, may be evidence of a mechanism of genome evolution used by ranaviruses.

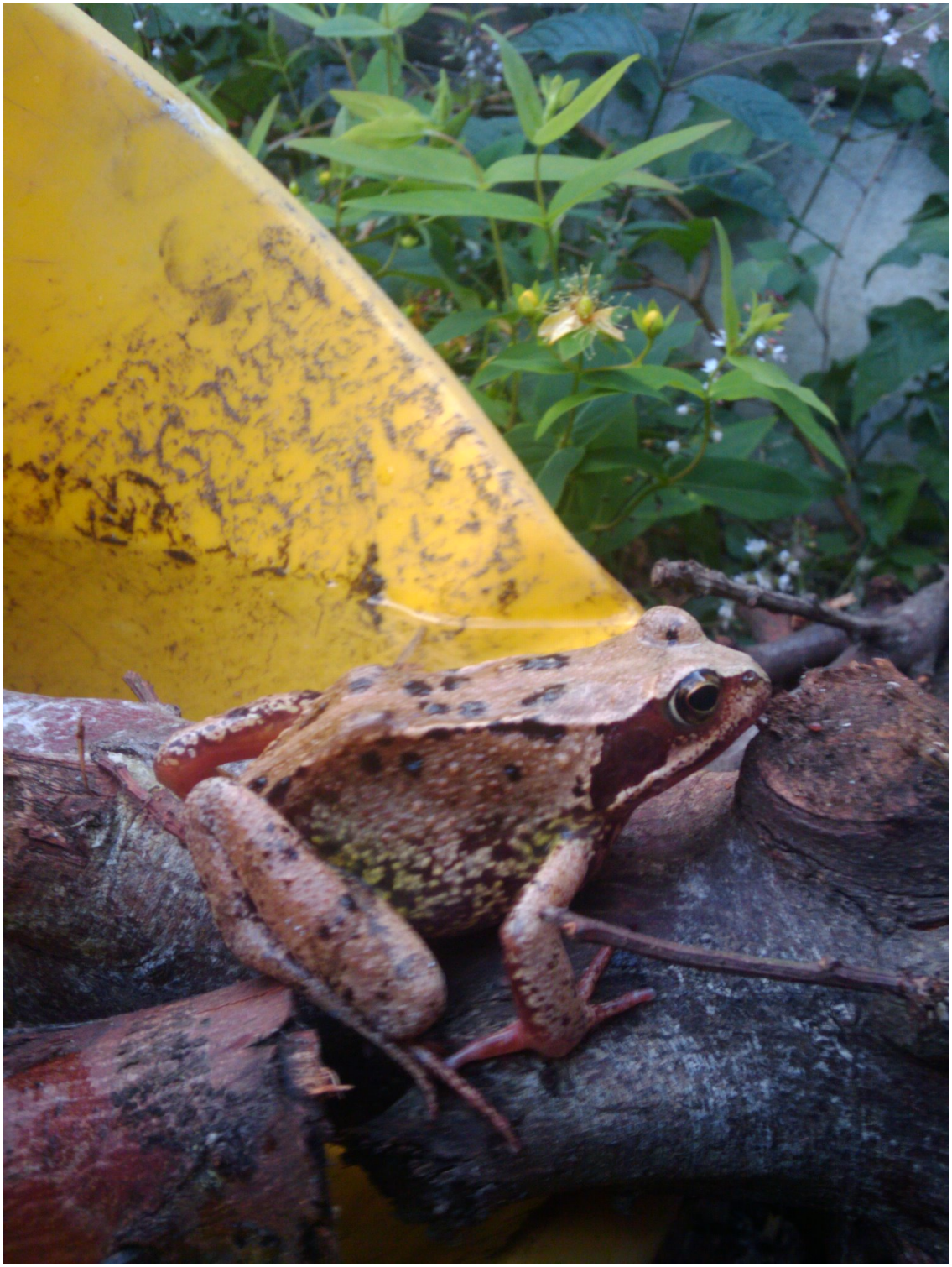
And one other key finding from the spatio-temporal modeling was a link between temperature and ranavirus emergence. An increased risk of ranavirus occurrence in warmer regions is consistent with observed seasonality and cell culture growth of ranaviruses in the laboratory and suggests that this preferred environmental envelope has influenced the pattern of the invasion of the UK. Seasonality of die-offs (Cunningham, 2001) and virus growth patterns in cell culture (Ariel et al., 2009) were a motivation for modeling climate effects on ranavirus spread though, if higher temperatures are contributing to ranavirus establishment or ranavirosis lethality, then the mechanism is interesting because environmental persistence of ranaviruses is enhanced at lower temps and indirect transmission is therefore more likely at lower temps (Nazir et al., 2012) and immune defense against ranavirus may be boosted at higher temperatures in Ambystomid salamanders (Rojas et al., 2005).

The contrast between Spain and the UK is stark. UK outbreaks have been focused in a single host species and in domestic garden ponds. In Spain, ranavirus outbreaks remain relatively restricted geographically but have occurred at multiple sites in a national park in addition to one man-made site. Furthermore the observed host range of viruses in Spain is extremely broad with overt disease and deaths in multiple hosts. However Spanish and UK ranaviruses do share an alarming trait and that is the ability to

cause significant host declines; several host species have undergone catastrophic decline in the Picos de Europa in just a few years. Smaller quantities of DNA sequence data were obtained from viruses in Spain, but sufficient for comparison of distinct isolates to provide considerable insight. Three distinct viruses were sampled and two of these had not previously been described. The phylogenetic connections between Spanish isolates and their relationship with the global ranavirus diversity suggests multiple introductions. Mapping the pattern of mortality, decline and host-use back to the phylogeny identifies the CMTV-like viruses as highly virulent pathogens with an extremely broad host range.

## Prospects

The study of UK ranavirus emergence would now benefit form a larger dataset covering a range of sites. The global spatial picture has been emerging slowly and whilst it is important for this to continue (and accelerate), there is a pressing need to drill down at a finer scale: sequencing of archived tissue samples and virus isolates would enable dated phylogenies to be constructed in order to date ancestral nodes, estimate rates of evolution and differences among lineages to be used to identify loci under selection during UK ranavirus emergence. In addition this more detailed data would yield further insights into the history of introduction and spread of these pathogens. Ranaviruses also offer potential as a model system to study evolutionary processes in dsDNA viruses, specifically in the context of evolutionary rates and speciation where the time series provided by the archived samples, and the recorded history of spread would be particularly valuable. Secondly their mechanisms of generating diversity may be revealing, and it could be particularly rewarding to follow up the evidence that the UK ranavirus has assimilated a bullfrog DNA sequence. Given their potential impacts on host populations and the consistent evidence for human intervention in their emergence, understanding the origin, dispersal and evolutionary dynamics of these pathogens should be considered a priority.



# Appendices

Appendix A: Post-mortem examination form

**PM ID** **Experiment ID**

Date of death Euthanized?
PM date:
Carcass condition: Sex: M / F / U
Body condition: Emaciated / thin / normal / fat
Weight: …….. g
Snout to vent length: …… mm
Left tibiofibular length…..…… mm
Photos taken: yes / no

**Gross Post Mortem Findings**
NE = not examined, NLD = no lesions detected, NA = not available
Where abnormalities exist, circle category and describe below:

| | | |
|---|---|---|
| 1 Integument | 6 Digestive | 11 Urinary |
| 2 Sensory | 7 Liver | 12 Endocrine |
| 3 Muscular | 8 Respiratory | 13 Reproductive |
| 4 Skeletal | 9 Cardiovascular | 14 Nervous |
| 5 Cavities | 10 Lymph-ret | |

**PM ID** **Experiment ID**

**PARASITOLOGY (specimens to be stored in 70% ethanol)**

Gross parasites Yes ☐ No ☐

Saved for identification? Yes ☐ No ☐

Results of identification

**MICROBIOLOGY**

Samples taken for *Bd* Swab ☐
PCR result Positive ☐ Negative ☐ Equivocal ☐

**RV SWABS**

Bd ☐ Buccal ☐

**HISTOLOGY (In 10% neutral buffered formalin)**

Liver ☐ Kidney ☐ Intestine ☐

Heart ☐ Muscle ☐ Skin ☐ Lungs ☐

Gonads ☐ Tongue ☐ Stomach ☐

Lesion ☐ If yes, where?

**SAMPLES FROZEN AT -20°C**

Liver ☐ Spleen ☐ Kidney ☐ Intestine ☐

Heart ☐ Muscle ☐ Skin ☐ Lungs ☐

Gonads ☐ Toe ☐ Tongue ☐ Stomach ☐

Lesion ☐ If yes, where?

**OTHER NOTES**

Appendix B: Primers used to join contigs in Chapter 4

| Primer name | Strand | FV3 start | Primer leng | Annealing | GC-content | Sequence | Product length |
|---|---|---|---|---|---|---|---|
| contig2_4_join | Forward | 10612 | 20 | 58.93 | 55 | GAAAGACTGCAAGCCTCGAG | 415 |
| contig2_4_join | Reverse | 11027 | 20 | 58.25 | 50 | CCCCACGCAGCTAAACATTA | 415 |
| contig4_12_join | Reverse | 22407 | 20 | 58.9 | 60 | CCTGTAAGTCGGTCCTCCTC | 370 |
| contig4_12_join | Forward | 22037 | 19 | 58.83 | 57.89 | CTGCAAGAGTCTTTCCCGC | 370 |
| contig12_9_join | Forward | 25682 | 18 | 59.43 | 66.67 | CTCGTCGTCCACCACCTC | 385 |
| contig12_9_join | Reverse | 26067 | 18 | 57.09 | 55.56 | TCGTCGGAGGAGCATTTG | 385 |
| contig9_18_join | Forward | 32256 | 20 | 58.91 | 50 | ATCCTCTTTTCTTTCGGCGC | 396 |
| contig9_18_join | Reverse | 32652 | 20 | 59.04 | 55 | CCCTGCACTTTTCCTTGACC | 396 |
| contig18_11_join | Forward | 33985 | 20 | 58.21 | 55 | GGATCTAGAAGACAGGGCCA | 390 |
| contig18_11_join | Reverse | 34375 | 19 | 59.25 | 63.16 | GTAGGTGGGGACGGCTATG | 390 |
| contig11_7_join | Forward | 38495 | 20 | 59.06 | 50 | GTCAAAACCTCGACGCTTGT | 891 |
| contig11_7_join | Reverse | 39386 | 18 | 59.3 | 61.11 | CAGTTGACGCGCCATAGC | 891 |
| contig7_10_join | Forward | 47138 | 20 | 58.81 | 55 | CTAAAGAGGCTGAGGTCCGT | 384 |
| contig7_10_join | Reverse | 47522 | 18 | 59.57 | 61.11 | GAACGTGCCTGGAACCCT | 384 |
| repeats_orf43 | Forward | 50759 | 19 | 59.12 | 57.89 | CAAATCGTGGTTGAGGCCC | 914 |
| repeats_orf43 | Reverse | 51673 | 20 | 58.49 | 50 | AGGTTGTGACTGTCAAGGGA | 914 |
| contig46_16_join | Forward | 53834 | 21 | 58.68 | 52.38 | GCATAGAGACGGATACAAGCG | 369 |
| contig46_16_join | Reverse | 54203 | 20 | 58.64 | 55 | GAAACAAGGCCGCTCTAGTC | 369 |
| contig16_97_join | Forward | 56623 | 20 | 59.17 | 60 | CCATGTACCCTCAGACCCTG | 389 |
| contig16_97_join | Reverse | 57012 | 20 | 58.99 | 55 | CATAGTCCGAACCCAAAGCG | 389 |
| contig97_6_join | Forward | 57668 | 20 | 58.56 | 50 | TTGAGGTCATTGTACGCAGC | 358 |
| contig97_6_join | Reverse | 58026 | 19 | 59.41 | 57.89 | AAGACCTGCTCGCTGAGAC | 358 |
| contig13_1_join | Forward | 68803 | 19 | 58.93 | 63.16 | GGGAGAGGGATGCCATACC | 388 |
| contig13_1_join | Reverse | 69191 | 18 | 60.04 | 66.67 | GGTGACTGTCATGGCCCC | 388 |
| contig1_8_join | Forward | 84726 | 20 | 59.82 | 55 | GGCCGACAATGACCTGAGAT | 397 |
| contig1_8_join | Reverse | 85123 | 20 | 59.46 | 55 | CAGAAAGAGGGCTCCGATGT | 397 |
| contig8_5_join | Forward | 91488 | 20 | 58.85 | 55 | CAGTCCGTGTCTGTCGTAGA | 399 |
| contig8_5_join | Reverse | 91887 | 20 | 58.76 | 55 | CTCCGAAAACACCCAGGTTC | 399 |
| contig5_2_join | Forward | 101328 | 19 | 59.25 | 57.89 | CTTCCCGTGTCTGGGTTGA | 374 |
| contig5_2_join | Reverse | 101702 | 20 | 58.9 | 55 | TGCACTCCGTAGCTCCTAAG | 374 |
| orf50l | Forward | 54391 | 21 | 58.76 | 52.38 | AGAGTCTGACTTGTCTGGGAC | 887 |
| orf50l | Reverse | 55278 | 21 | 57.01 | 42.86 | TGCAAGCAGAGGATAAAGTCT | 887 |
| fv3_orf26r | Forward | 32805 | 21 | 57.6 | 42.86 | AACAAGTATTCGGCAGACACT | 391 |
| fv3_orf26r | Reverse | 33196 | 20 | 59.89 | 55 | TACACAAAGGGGCACAGTCC | 391 |
| contig2_fusion | Forward | 10339 | 20 | 59.1 | 60 | GGGACACTCTACCTGAACCC | 884 |
| contig2_fusion | Reverse | 11223 | 20 | 59.31 | 55 | CCTGCCAACCTTGTCCTAGT | 884 |
| contig10_46_join | Forward | 52410 | 20 | 59.04 | 50 | TGGCCATGAAACAGACTTGC | 420 |
| contig10_46_join | Reverse | 52830 | 21 | 59.11 | 47.62 | TCATCTCCCGTCGTGAAAAGA | 420 |
| contig6_13_asFV3 | Forward | 65228 | 19 | 59.85 | 57.89 | TCAAGGTGTGCAGGGACAC | 892 |
| contig6_13_asFV3 | Reverse | 66120 | 21 | 58.72 | 47.62 | AGGGTGGTAATCTTTCAGGCT | 892 |
| contig18_insert_start | Forward | 248 | 21 | 57.6 | 42.86 | AACAAGTATTCGGCAGACACT | 379 |
| contig18_insert_start | Reverse | 627 | 23 | 57.5 | 39.13 | ACTCCTTGCaAAAGTATACCTCA | 379 |
| contig18_insert_end | Forward | 1024 | 23 | 58.62 | 47.83 | GGAATAAAGGGATCTGCAGTCTC | 418 |
| contig18_insert_end | Reverse | 1442 | 20 | 59.89 | 55 | TACACAAAGGGGCACAGTCC | 418 |
| contig6_13_ruk13insert | Forward | 8345 | 20 | 57.26 | 45 | TGTTCTGCCTAAACCAACCA | 868 |
| contig6_13_ruk13insert | Reverse | 9213 | 20 | 58.84 | 55 | GCTGCAAGTCTGGGAGAAAG | 868 |

Appendix C: raw data for Fisher tests of association between host declines and infection with CMTV-like viruses

| site | Die offs and/or CMTV absent | | CMTV present | |
|---|---|---|---|---|
| | No decline | Declining | No decline | Declining |
| Ándara lake | 1 | 0 | 0 | 0 |
| Bajero lake | 2 | 0 | 0 | 0 |
| Charcas de Cable | 2 | 0 | 0 | 0 |
| El Pontón | 1 | 0 | 0 | 0 |
| Ercina lake | 0 | 0 | 0 | 1 |
| La Güelga | 1 | 0 | 0 | 0 |
| Lloroza | 0 | 0 | 1 | 1 |
| Moñetas | 0 | 0 | 0 | 2 |
| Orandi-Covadonga road | 1 | 0 | 0 | 0 |
| Pontón-Oseja road | 2 | 0 | 0 | 0 |
| Pozu Llau | 2 | 1 | 0 | 0 |
| Rasa Pandecarmen | 1 | 0 | 0 | 0 |
| Soto-Covadonga road | 0 | 1 | 0 | 0 |
| Vau los Lobos | 1 | 0 | 0 | 0 |
| Vega Salambre | 1 | 0 | 0 | 0 |
| TOTAL: individual hosts | 15 | 2 | 1 | 4 |
| TOTAL: sites | 10 | 2 | 0 | 3 |

Appendix D: FBA news article

# Citizen Science: The Frog Mortality Project and Beyond

*Stephen Price is a molecular ecologist who just happens to work on an aquatic problem, studying the spread of a frog pathogen (ranavirus) in the UK for his PhD supervised by Dr Trenton Garner (Institute of Zoology) and Prof Richard Nichols (QMUL). One of the tools at his disposal is a huge database of reports of unusual amphibian mortality, which he discusses here in the broader context of citizen science projects.*

Large scale public participation in scientific research began at the start of the 20th century with the Christmas bird count in the USA, and 'twitchers' were again the trailblazers in the UK: the British Trust for Ornithology was established in 1933 to harness the energy of enthusiasts for the benefit of science and conservation[1].

We are now in the midst of an explosion in Citizen Science projects thanks to the internet and smart phones, scientists finally realising the resource that the public represents, and outreach commitments to funding bodies[2].

Existing projects (see Fig. 1 for examples) can involve data collection for surveys/phenology studies or data interpretation by offering up spare brain or computer capacity (distributed thinking/computing).

## The Frog Mortality Project (FMP): surveillance by the public

The Zoological Society of London (ZSL) and others received increasing numbers of unsolicited reports of unusual frog mortality from 1985 onwards. The FMP was established in 1992 to collect, collate and respond to these reports. It therefore pre-dates the citizen science explosion and presents a good opportunity to assess benefits, pitfalls and points to consider in this type of amateur-professional collaboration.

A *Ranavirus* (see Box 1) was later identified as the aetiological agent of a fatal amphibian disease consistent with many FMP reports[6]. Key events in FMP history are shown in Fig. 2. After 1992, records were sought through the media and word of mouth with electronic records – collated in Microsoft Access (a relational database management system) – taking over from paper by 2001. By 2009 paper reports had been assimilated into the

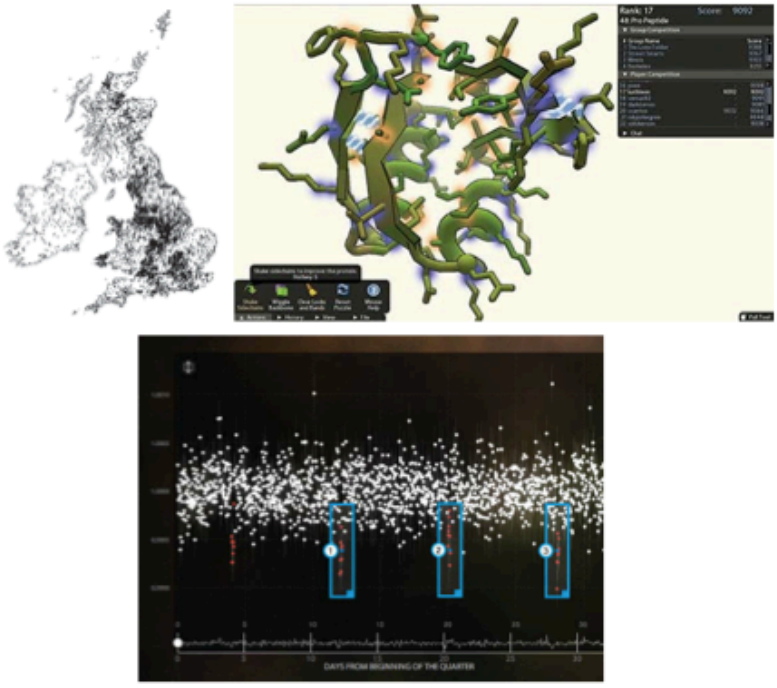


Figure 1. Examples of citizen science projects; (top left) *Rana temporaria* (Common frog) distribution map generated via NBN[3], (bottom) example light emission graph analysed by would-be planethunters[4], (top right) a protein building puzzle from Foldit[5]

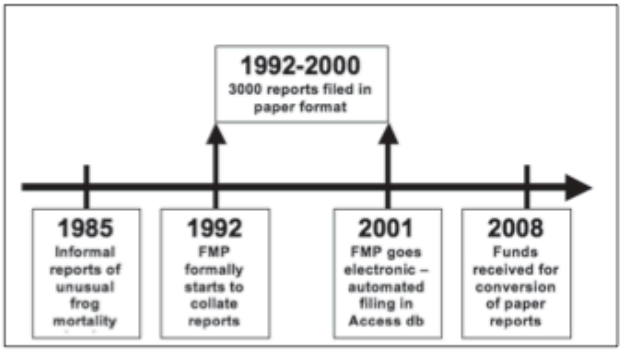



Figure 2. Timeline of key events in FMP reporting and data management

electronic database and records were divided into tables according to the year the report was filed, and subdivided based on the likelihood of ranavirus being the cause of mortality.

## ASSESSING THE FMP

### Data, data, data...

The FMP's most obvious strength is size; by 2010 it was home to 4845 reports with information in up to 169 fields. Acquiring such a huge volume of records was made possible through public participation whilst the large number of fields carries potential for a broad range of study questions (around amphibian health and beyond). Data cover site information, the mortality event, the pond and its setting, and more besides.

Unfortunately the ultimate use and quality of data was not considered adequately when choosing either the software or its implementation. The relational capacity of Access tables is not utilised and the inaccurate and somewhat arbitrary splitting of records into tables is unhelpful. The conversion of records from a paper to an electronic format was, however, absolutely necessary. Mistakes based on misreading, misunderstanding or mistyping are practically unavoidable but quality checking was insufficient and unnecessary errors were introduced because 'tickable' boxes – easily altered by the accidental press of a button – were used to represent nominal variables.

Ranavirus is an emerging infectious disease in the UK following likely introduction from North America[7]. The pattern of spread is a current research priority and the FMP database is an obvious resource in this respect. Fields allowing georeferencing (e.g. postcode) and unique identification of sites are essential. However the database has a lot of missing data; for example, 5.4% of reports either have no postcode or have an error therein, precluding georeferencing.

### Constraint versus free rein?

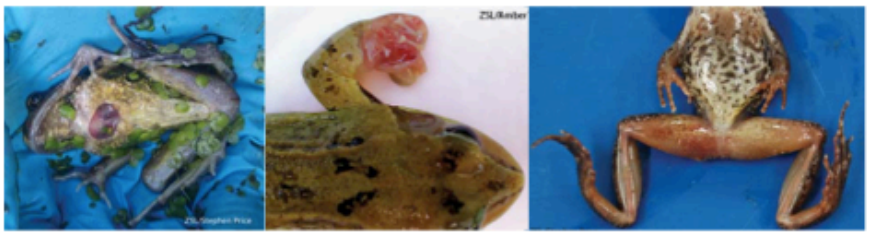
Questionnaire responses highlight the dilemma between giving reporters free rein and controlling responses tightly. The free rein approach introduces lots of miscellaneous and (sometimes) useless data. When asked for a number of deaths one in 25 responses were not integers but rather ranges or descriptive terms not amenable to analysis without transformation by interpretation.

On the other hand, when questioned on abnormalities ('signs') present at death, "yes/no" answers were solicited to indicate presence/absence. Initially this appears adequate but, given that many reports relate to multiple mortalities, we have no idea whether a given sign was observed in one or all of the carcasses. In addition, offering up multiple-choice answers encourages reporters to guess.

### Box 1. Ranavirus Disease

Ranaviruses are large, double stranded DNA viruses infecting amphibians, reptiles and fish. They have a global distribution and are now widespread in England following likely introduction from North America.

They are highly virulent pathogens; infection can lead to a nasty disease which kills many thousands of adult common frogs in the UK each year, whilst infections are also documented in other host species.



Common frogs with (left to right) severe ulceration, extreme limb necrosis and mild skin reddening

Infection at a site often results in multiple mortalities from late spring through summer and infection may recur from one year to the next. Animals may suffer ulceration, loss of digits/limbs, systemic haemorrhaging resulting in bleeding from the mouth or anus, skin reddening (especially the thighs), lethargy and emaciation but may also appear asymptomatic.

Further information is available at http://www.froglife.org/disease/frog_mortality_project.htm where unusual amphibian mortalities can also be reported.

### Do the public always...

#### ... 1. make precise measurements?

Repeat reporters to the FMP reveal significant discrepancies in size estimation. When comparing pond dimensions between reports from the same pond, discrepancies of more than 10% in one or more dimensions were seen in 97% of cases. Actual pond dimensions can vary over time although even after discounting depth (the more likely dimension to change) 87% of reporters still report differences of more than 10%.

#### 2. ...look closely?

The public may rarely undertake thorough assessments of decomposing carcasses, undermining assertions relating to subtle signs of disease. In a survey of eight ponds where recurrent mortality events with overt ranavirus-consistent signs had been reported, on-going or healed lesions were found at each[8]. This suggests consistent, accurate reporting of signs but makes no assessment of under-reporting. Estimates of healthy frog population size are also sought but, without training or knowledge of the animal's ecology, this is unlikely to elicit accurate or even *consistently inaccurate* responses.

#### 3. ...answer honestly?

The FMP asks about pond management (usually undertaken by reporters themselves); e.g. pesticides and water treatments used in the pond or vicinity, and movement of animals/plants in or out. It is easy for reporters to assume that these practices contribute to the mortality event by some means and having made this inference it's unlikely that everyone will answer honestly. Anecdotal evidence confirms that they don't: one very helpful reporter stated that he never moves spawn or tadpoles from his pond though his wife later confided; "there's sometimes so much spawn that my husband moves some to the park"!

### Prospects: public help to do good science

Future projects of this type would benefit from thorough planning in the following areas:

a. **Formulating questions**
The establishment of the FMP in response to unsolicited reports of mortality (about which nothing was known) meant that there was a lack of strategic organisation. Reporters initially guided database content – to some extent the information available defined fields. Fundamental and interesting questions about disease, e.g. the prevalence of particular signs, are impossible to answer due to the manner of data collection and organisation. These points underline the importance of having specific questions (even simple ones) in place before data collection commences.

b. **Questionnaire and IT design**
Careful planning regarding data management and how to ask research questions in order to elicit useful answers are required at the outset. Software capacity and functionality should be considered thoroughly in consultation with experts in order to match them to requirements and avoid unnecessary barriers to analysis. Questionnaire design is a key issue if the right balance is to be struck between constraint and free rein. Biologists may profit here from collaboration with medics and psychologists who have much experience backed up by a rich literature.

c. **Know your public: training, direction, validation and corrections**
It is essential to consider the capabilities of citizens in relation to the data they are asked to gather. Is training (or at least instruction) required to gather reliable data? How are reports validated? Is there a way to apply corrections?

Ranavirus may be inextricably linked to garden ponds as a result of some human intervention and if so research is absolutely dependent on pond-owners. Either way, the FMP has certainly benefited from the contributions of extremely conscientious citizen scientists and is a great example of citizens mobilising scientists rather than vice-versa.

The FMP is a pioneering citizen science project so it's appropriate that it should help guide future strategy in this area. Clearly, thorough planning is required if one is to maximise data. The impact of collaborations with experts in data management and questionnaire design at the outset should not be underestimated.

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**References**

1 **About BTO - History** [www.bto.org/about-bto/history]
2 Silvertown J. **A new dawn for citizen science.** Trends in Ecology & Evolution 2009:467-471
3 **National Biodiversity Network** [www.nbn.org.uk/Home.aspx]
4 **Planethunters** [www.planethunters.org/]
5 **Fold it** [http://fold.it/portal/]
6 Cunningham et al., 1996. **Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*).** Philosophical Transactions of the Royal Society, London B. 351: 1539-1557
7 Hyatt et al., 2000. **Comparative studies of piscine and amphibian iridoviruses.** Arch Virol (2000) 145: 301–331
8 Teacher, A., 2008. **Population and Immunocompetent Genetic Variation: A Field-Based Study.** Queen Mary, University of London School of Biological and Chemical Sciences

## Freshwater Reviews – call for volunteer copy-editors and proofreaders

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# References

Ahne, W., Bremont, M., Hedrick, R.P., Hyatt, A.D., Whittington, R.J., 1997. Iridoviruses associated with epizootic haematopoietic necrosis (EHN) in aquaculture. World Journal of Microbiology & Biotechnology 13, 367–373.

Ahne, W., Schlotfeldt, H.J., Thomsen, I., 1989. Fish viruses: isolation of an icosahedral cytoplasmic deoxyribovirus from sheatfish (*Silurus glanis*). Zentralblatt fur Veterinarmedizin. Reihe B. Journal of veterinary medicine. Series B 36, 333–6.

Alcami, A., Koszinowski, U.H., 2000. Viral mechanisms of immune evasion. Immunol. Today 21, 447–455.

Alford, R.A., Richards, S.J., 1999. Global amphibian declines: A problem in applied ecology. Annu. Rev. Ecol. Syst. 30, 133–165.

Altizer, S., Harvell, D., Friedle, E., 2003. Rapid evolutionary dynamics and disease threats to biodiversity. Trends Ecol. Evol. 18, 589–596.

Alves de Matos, A.P., da Silva Trabucho Caeiro, M.F.A., Papp, T., da Cunha Almeida Matos, B.A., Lacerda Correia, A.C., Marschang, R.E., 2011. New Viruses from Lacerta monticola (Serra da Estrela, Portugal): Further Evidence for a New Group of Nucleo-Cytoplasmic Large Deoxyriboviruses. Microscopy and Microanalysis 17, 101–108.

Ariel, E., Holopainen, R., Olesen, N.J., Tapiovaara, H., 2010. Comparative study of ranavirus isolates from cod (*Gadus morhua*) and turbot (*Psetta maxima*) with reference to other ranaviruses. Archives of Virology 155, 1261–1271.

Ariel, E., Kielgast, J., Svart, H.E., Larsen, K., Tapiovaara, H., Jensen, B.B., Holopainen, R., 2009. Ranavirus in wild edible frogs *Pelophylax kl. esculentus* in Denmark. Diseases of Aquatic Organisms 85, 7–14.

Ariel, E., Nicolajsen, N., Christophersen, M.-B., Holopainen, R., Tapiovaara, H., Jensen, B.B., 2009. Propagation and isolation of ranaviruses in cell culture. Aquaculture 294, 159–164.

Balseiro, A., Dalton, K.P., del Cerro, A., Marquez, I., Cunningham, A.A., Parra, F., Prieto, J.M., Casais, R., 2009. Pathology, isolation and molecular characterisation of a ranavirus from the common midwife toad *Alytes obstetricans* on the Iberian Peninsula. Diseases of Aquatic Organisms 84, 95–104.

Balseiro, A., Dalton, K.P., del Cerro, A., Marquez, I., Parra, F., Prieto, J.M., Casais, R., 2010. Outbreak of common midwife toad virus in alpine newts (*Mesotriton alpestris cyreni*) and common midwife toads (*Alytes obstetricans*) in Northern Spain A comparative pathological study of an emerging ranavirus. Veterinary Journal 186, 256–258.

Banoo, S., Bell, D., Bossuyt, P., Herring, A., Mabey, D., Poole, F., Smith, P.G., Sriram, N., Wongsrichanalai, C., Linke, R., O'Brien, R., Perkins, M., Cunningham, J., Matsoso, P., Nathanson, C.M., Olliaro, P., Peeling, R.W., Ramsay, A., 2008. Evaluation of diagnostic tests for infectious diseases: general principles. Nat Rev Micro 8, S16–S28.

Bayley, A.E., Hill, B.J., Feist, S.W., 2013. Susceptibility of the European common frog *Rana temporaria* to a panel of ranavirus isolates from fish and amphibian hosts. Diseases of aquatic organisms 103, 171–83.

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H., 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc. Natl. Acad. Sci. U. S. A. 95, 9031–9036.

Bratke, K.A., McLysaght, A., 2008. Identification of multiple independent horizontal gene transfers into poxviruses using a comparative genomics approach. Bmc Evol. Biol. 8, 67.

Brunner, J.L., Richards, K., Collins, J.P., 2005. Dose and host characteristics influence virulence of ranavirus infections. Oecologia 144, 399–406.

Brunner, J.L., Schock, D.M., Collins, J.P., 2007. Transmission dynamics of the amphibian ranavirus *Ambystoma tigrinum virus*. Diseases of Aquatic Organisms 77, 87–95.

Brunner, J.L., Schock, D.M., Davidson, E.W., Collins, J.P., 2004. Intraspecific reservoirs: Complex life history and the persistence of a lethal ranavirus. Ecology 85, 560–566.

Chen, G., Ward, B.M., Yu, K.H., Chinchar, V.G., Robert, J., 2011. Improved Knockout Methodology Reveals That *Frog virus 3* Mutants Lacking either the 18K Immediate-Early Gene or the Truncated vIF-2 alpha Gene Are Defective for Replication and Growth In Vivo. Journal of Virology 85, 11131–11138.

Chen, Z.X., Zheng, J.C., Jiang, Y.L., 1999. A new iridovirus isolated from soft-shelled turtle. Virus Research 63, 147–151.

Chinchar, V.G., 2002. Ranaviruses (family *Iridoviridae*): emerging cold-blooded killers - Brief review. Archives of Virology 147, 447–470.

Chinchar, V.G., Mao, J.H., 2000. Molecular diagnosis of iridovirus infections in cold-blooded animals. Seminars in Avian and Exotic Pet Medicine 9, 27–35.

Chinchar, V.G., Yu, K.H., Jancovich, J.K., 2011. The Molecular Biology of *Frog virus 3* and other Iridoviruses Infecting Cold-Blooded Vertebrates. Viruses-Basel 3, 1959–1985.

Cleaveland, S., Laurenson, M.K., Taylor, L.H., 2001. Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci. 356, 991–999.

Collins, J.P., Storfer, A., 2003. Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9, 89–98.

Cullen, B.R., Owens, L., 2002. Experimental challenge and clinical cases of *Bohle iridovirus* (BIV) in native Australian anurans. Diseases of Aquatic Organisms 49, 83–92.

Cunningham, A.A., 1996. Disease risks of wildlife translocations. Conserv. Biol. 10, 349–353.

Cunningham, A.A., 2001. Investigations into mass mortalities of the common frog (*Rana temporaria*) in Britain: epidemiology and aetiology. Royal Veterinary College (University of London).

Cunningham, A.A., Daszak, P., Rodriguez, J.P., 2003. Pathogen pollution: defining a parasitological threat to biodiversity conservation. J. Parasitol. 89, S78–S83.

Cunningham, A.A., Dobson, A.P., Hudson, P.J., 2012. Disease invasion: impacts on biodiversity and human health Introduction. Philos. Trans. R. Soc. B-Biol. Sci. 367, 2804–2806.

Cunningham, A.A., Garner, T.W.J., Aguilar-Sanchez, V., Banks, B., Foster, J., Sainsbury, A.W., Perkins, M., Walker, S.F., Hyatt, A.D., Fisher, M.C., 2005. Emergence of amphibian chytridiomycosis in Britain. Veterinary Record 157, 386–387.

Cunningham, A.A., Hyatt, A.D., Russell, P., Bennett, P.M., 2007a. Emerging epidemic diseases of frogs in Britain are dependent on the source of ranavirus agent and the route of exposure. Epidemiology and Infection 135, 1200–1212.

Cunningham, A.A., Hyatt, A.D., Russell, P., Bennett, P.M., 2007b. Experimental transmission of a ranavirus disease of common toads (*Bufo bufo*) to common frogs (*Rana temporaria*). Epidemiology and Infection 135, 1213–1216.

Cunningham, A.A., Langton, T.E.S., Bennett, P.M., Lewin, J.F., Drury, S.E.N., Gough, R.E., MacGregor, S.K., 1996. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 351, 1539–1557.

Cunningham, A.A., Tems, C.A., Russell, P.H., 2008. Immunohistochemical demonstration of ranavirus antigen in the tissues of infected frogs (*Rana temporaria*) with systemic haemorrhagic or cutaneous ulcerative disease. Journal of Comparative Pathology 138, 3–11.

Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E., Speare, R., 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5, 735–748.

Daszak, P., Cunningham, A.A., Hyatt, A.D., 2000. Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. Science 287, 443–449.

Daszak, P., Cunningham, A.A., Hyatt, A.D., 2003. Infectious disease and amphibian population declines. Divers. Distrib. 9, 141–150.

Dobson, A., Foufopoulos, J., 2001. Emerging infectious pathogens of wildlife. Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci. 356, 1001–1012.

Duffus, A.L.J., 2009. Chytrid Blinders: What Other Disease Risks to Amphibians Are We Missing? Ecohealth 6, 335–339.

Duffus, A.L.J., Cunningham, A.A., 2010. Major disease threats to European amphibians. Herpetological Journal 20, 117–127.

Eaton, H.E., Metcalf, J., Penny, E., Tcherepanov, V., Upton, C., Brunetti, C.R., 2007. Comparative genomic analysis of the family *Iridoviridae*: re-annotating and defining the core set of iridovirus genes. Virology Journal 4, 11.

Eaton, H.E., Ring, B.A., Brunetti, C.R., 2010. The Genomic Diversity and Phylogenetic Relationship in the Family *Iridoviridae*. Viruses-Basel 2, 1458–1475.

Ebert, D., 1998. Evolution - Experimental evolution of parasites. Science 282, 1432–1435.

Echaubard, P., Little, K., Pauli, B., Lesbarreres, D., 2010. Context-Dependent Effects of Ranaviral Infection on Northern Leopard Frog Life History Traits. PLoS One 5 (10), e13723.

Essbauer, S., Bremont, M., Ahne, W., 2001. Comparison of the eIF-2α Homologous Proteins of Seven Ranaviruses (*Iridoviridae*). Virus Genes 23, 347–359.

Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., Cunningham, A.A., Weldon, C., du Preez, L.H., Anderson, L., Pond, S.L.K., Shahar-Golan, R., Henk, D.A., Fisher, M.C., 2011. Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. Proceedings of the National Academy of Sciences of the United States of America 108, 18732–18736.

Fijan N, Matasin Z, Petrinec Z, Valpotiç I, Zwillenberg LO, 1991. Isolation of an iridovirus-like agent from the green frog (*Rana esculenta L.*). Vet Arch Zagreb 3, 151–158.

Filée, J., 2009. Lateral gene transfer, lineage-specific gene expansion and the evolution of Nucleo Cytoplasmic Large DNA viruses. J. Invertebr. Pathol. 101, 169–171.

Filée, J., Chandler, M., 2008. Convergent mechanisms of genome evolution of large and giant DNA viruses. Research in Microbiology 159, 325–331.

Firth, C., Kitchen, A., Shapiro, B., Suchard, M.A., Holmes, E.C., Rambaut, A., 2010. Using Time-Structured Data to Estimate Evolutionary Rates of Double-Stranded DNA Viruses. Mol. Biol. Evol. 27, 2038–2051.

Fisher, M.C., 2007. Potential interactions between amphibian immunity, infectious disease and climate change. Animal Conservation 10, 420–421.

Fisher, M.C., Garner, T.W.J., Walker, S.F., 2009. Global Emergence of *Batrachochytrium dendrobatidis* and Amphibian Chytridiomycosis in Space, Time, and Host. Annual Review of Microbiology. 63, 291–310.

Fox, S.F., Greer, A.L., Torres-Cervantes, R., Collins, J.P., 2006. First case of ranavirus-associated morbidity and mortality in natural populations of the South American frog *Atelognathus patagonicus*. Diseases of Aquatic Organisms 72, 87–92.

Frick, W.F., Pollock, J.F., Hicks, A.C., Langwig, K.E., Reynolds, D.S., Turner, G.G., Butchkoski, C.M., Kunz, T.H., 2010. An Emerging Disease Causes

Regional Population Collapse of a Common North American Bat Species. Science 329, 679–682.

Garner, T.W.J., Briggs, C.J., Bielby, J., Fisher, M.C., 2012. Determining When Parasites of Amphibians are Conservation Threats to Their Hosts, in: New Directions in Conservation Medicine: Applied Cases of Ecological Health. pp. 521–538.

Go, J., Lancaster, M., Deece, K., Dhungyel, O., Whittington, R., 2006. The molecular epidemiology of iridovirus in Murray cod (*Maccullochella peelii peelii*) and dwarf gourami (*Colisa lalia*) from distant biogeographical regions suggests a link between trade in ornamental fish and emerging iridoviral diseases. Molecular and Cellular Probes 20, 212–222.

Granoff, A., Came, P.E., Breeze, D.C., 1966. Viruses and renal carcinoma of *Rana pipiens*. I. The isolation and properties of virus from normal and tumor tissue. Virology 29, 133–48.

Gray, M.J., Miller, D.L., Hoverman, J.T., 2009. Ecology and pathology of amphibian ranaviruses. Diseases of Aquatic Organisms 87, 243–266.

Gray, M.J., Miller, D.L., Hoverman, J.T., 2012. Reliability of non-lethal surveillance methods for detecting ranavirus infection. Diseases of Aquatic Organisms 99, 1–6.

Gray, M.J., Miller, D.L., Schmutzer, A.C., Baldwin, C.A., 2007. *Frog virus 3* prevalence in tadpole populations inhabiting cattle-access and non-access wetlands in Tennessee, USA. Diseases of Aquatic Organisms 77, 97–103.

Green, D.E., Converse, K.A., Schrader, A.K., 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Ann. N. Y. Acad. Sci. 969, 323–339.

Greer, A.L., Berrill, M., Wilson, P.J., 2005. Five amphibian mortality events associated with ranavirus infection in south central Ontario, Canada. Diseases of Aquatic Organisms 67, 9–14.

Greer, A.L., Collins, J.P., 2007. Sensitivity of a diagnostic test for amphibian ranavinus varies with sampling protocol. Journal of Wildlife Diseases 43, 525–532.

Grenfell, B.T., Pybus, O.G., Gog, J.R., Wood, J.L.N., Daly, J.M., Mumford, J.A., Holmes, E.C., 2004. Unifying the epidemiological and evolutionary dynamics of pathogens. Science 303, 327–332.

Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., Samuel, M.D., 2002. Ecology - Climate warming and disease risks for terrestrial and marine biota. Science 296, 2158–2162.

Hay, J., Ruvinsky, I., Hedges, S., Maxson, L., 1995. Phylogenetic-Relationships of Amphibian Families Inferred from Dna-Sequences of Mitochondrial 12s and 16s Ribosomal-Rna Genes. Molecular Biology and Evolution 12, 928–937.

He, J.G., Lu, L., Deng, M., He, H.H., Weng, S.P., Wang, X.H., Zhou, S.Y., Long, Q.X., Wang, X.Z., Chan, S.M., 2002. Sequence analysis of the complete genome of an iridovirus isolated from the tiger frog. Virology 292, 185–197.

Hedrick, R., Mcdowell, T., 1995. Properties of Iridoviruses from Ornamental Fish. Veterinary Research 26, 423–427.

Hellsten, U., Harland, R.M., Gilchrist, M.J., Hendrix, D., Jurka, J., Kapitonov, V., Ovcharenko, I., Putnam, N.H., Shu, S., Taher, L., Blitz, I.L., Blumberg, B., Dichmann, D.S., Dubchak, I., Amaya, E., Detter, J.C., Fletcher, R., Gerhard, D.S., Goodstein, D., Graves, T., Grigoriev, I.V., Grimwood, J., Kawashima, T., Lindquist, E., Lucas, S.M., Mead, P.E., Mitros, T., Ogino, H., Ohta, Y., Poliakov, A.V., Pollet, N., Robert, J., Salamov, A., Sater, A.K., Schmutz, J., Terry, A., Vize, P.D., Warren, W.C., Wells, D., Wills, A., Wilson, R.K., Zimmerman, L.B., Zorn, A.M., Grainger, R., Grammer, T., Khokha, M.K., Richardson, P.M., Rokhsar, D.S., 2010. The Genome of the Western Clawed Frog *Xenopus tropicalis*. Science 328, 633–636.

Hitchings, S.P., Beebee, T.J.C., 1997. Genetic substructuring as a result of barriers to gene flow in urban *Rana temporaria* (common frog) populations: implications for biodiversity conservation. Heredity 79, 117–127.

Holmes, E., Nee, S., Rambaut, A., Garnett, G., Harvey, P., 1995. Revealing the History of Infectious-Disease Epidemics Through Phylogenetic Trees. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 349, 33–40.

Holmes, E.C., 1998. Molecular epidemiology and evolution of emerging infectious diseases. British Medical Bulletin 54, 533–543.

Holopainen, R., Honkanen, J., Jensen, B.B., Ariel, E., Tapiovaara, H., 2011. Quantitation of ranaviruses in cell culture and tissue samples. Journal of Virological Methods 171, 225–233.

Huang, Y., Huang, X., Liu, H., Gong, J., Ouyang, Z., Cui, H., Cao, J., Zhao, Y., Wang, X., Jiang, Y., Qin, Q., 2009. Complete sequence determination of a novel reptile iridovirus isolated from soft-shelled turtle and evolutionary analysis of *Iridoviridae*. BMC Genomics 10, 224.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Hughes, A.L., Irausquin, S., Friedman, R., 2010. The evolutionary biology of poxviruses. Infect. Genet. Evol. 10, 50–59.

Hyatt, A.D., Gould, A.R., Zupanovic, Z., Cunningham, A.A., Hengstberger, S., Whittington, R.J., Kattenbelt, J., Coupar, B.E.H., 2000. Comparative studies of piscine and amphibian iridoviruses. Archives of Virology 145, 301–331.

Hyatt, A.D., Williamson, M., Coupar, B.E.H., Middleton, D., Hengstberger, S.G., Gould, A.R., Selleck, P., Wise, T.G., Kattenbelt, J., Cunningham, A.A., Lee, J., 2002. First identification of a ranavirus from green pythons (*Chondropython viridis*). Journal of Wildlife Diseases 38, 239–252.

Jancovich, J.K., Bremont, M., Touchman, J.W., Jacobs, B.L., 2010. Evidence for Multiple Recent Host Species Shifts among the Ranaviruses (Family *Iridoviridae*). Journal of Virology 84, 2636–2647.

Jancovich, J.K., Davidson, E.W., Morado, J.F., Jacobs, B.L., Collins, J.P., 1997. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. Diseases of Aquatic Organisms 31, 161–167.

Jancovich, J.K., Davidson, E.W., Parameswaran, N., Mao, J., Chinchar, V.G., Collins, J.P., Jacobs, B.L., Storfer, A., 2005. Evidence for emergence of an amphibian iridoviral disease because of human-enhanced spread. Molecular Ecology 14, 213–224.

Jancovich, J.K., Davidson, E.W., Seiler, A., Jacobs, B.L., Collins, J.P., 2001. Transmission of the *Ambystoma tigrinum virus* to alternative hosts. Diseases of Aquatic Organisms 46, 159–163.

Jeong, J.B., Kim, H.Y., Jun, L.J., Lyu, A.H., Park, N.G., Kim, J.K., Do Jeong, H., 2008. Outbreaks and risks of infectious spleen and kidney necrosis virus disease in freshwater ornamental fishes. Diseases of Aquatic Organisms 78, 209–215.

Johnson, P.T.J., Rohr, J.R., Hoverman, J.T., Kellermanns, E., Bowerman, J., Lunde, K.B., 2012. Living fast and dying of infection: host life history drives

interspecific variation in infection and disease risk. Ecology Letters 15, 235–242.

Katoh, K., Standley, D.M., 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Mol. Biol. Evol. 30, 772–780.

Kik, M., Martel, A., Spitzen-van der Sluijs, A., Pasmans, F., Wohlsein, P., Grone, A., Rijks, J.M., 2011. Ranavirus-associated mass mortality in wild amphibians, The Netherlands, 2010: A first report. Veterinary Journal 190, 284–286.

Kirzinger, M.W.B., Stavrinides, J., 2012. Host specificity determinants as a genetic continuum. Trends Microbiol. 20, 88–93.

Koonin, E.V., Dolja, V.V., 2006. Evolution of complexity in the viral world: The dawn of a new vision. Virus Res. 117, 1–4.

Korbie, D.J., Mattick, J.S., 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nature Protocols 3, 1452–1456.

LaDeau, S.L., Kilpatrick, A.M., Marra, P.P., 2007. West Nile virus emergence and large-scale declines of North American bird populations. Nature 447, 710–713.

Langdon, J., Humphrey, J., Williams, L., Hyatt, A., Westbury, H., 1986. 1st Virus Isolation from Australian Fish - an Iridovirus-Like Pathogen from Redfin Perch, *Perca fluviatilis* L. Journal of Fish Diseases 9, 263–268.

Langton, T.E.S., Atkins, W., Herbert, C., 2011. On the distribution, ecology and management of non-native reptiles and amphibians in the London area. Part 1. Distribution and predatory/prey impacts. London Naturalist 90, 83–155.

Lawson, B., Lachish, S., Colvile, K.M., Durrant, C., Peck, K.M., Toms, M.P., Sheldon, B.C., Cunningham, A.A., 2012. Emergence of a Novel Avian Pox Disease in British Tit Species. PLoS One 7, e40176.

Lawson, B., Robinson, R.A., Colvile, K.M., Peck, K.M., Chantrey, J., Pennycott, T.W., Simpson, V.R., Toms, M.P., Cunningham, A.A., 2012. The emergence and spread of finch trichomonosis in the British Isles. Philosophical Transactions of the Royal Society B-Biological Sciences 367, 2852–2863.

Lei, X.Y., Ou, T., Zhu, R.L., Zhang, Q.Y., 2012. Sequencing and analysis of the complete genome of *Rana grylio virus* (RGV). Archives of Virology 157, 1559–1564.

Lips, K.R., Brem, F., Brenes, R., Reeve, J.D., Alford, R.A., Voyles, J., Carey, C., Livo, L., Pessier, A.P., Collins, J.P., 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Sciences of the United States of America 103, 3165–3170.

Loytynoja, A., Goldman, N., 2005. An algorithm for progressive multiple alignment of sequences with insertions. Proc. Natl. Acad. Sci. U. S. A. 102, 10557–10562.

Maniero, G.D., Morales, H., Gantress, J., Robert, J., 2006. Generation of a long-lasting, protective, and neutralizing antibody response to the ranavirus FV3 by the frog *Xenopus*. Developmental and Comparative Immunology 30, 649–657.

Mao, J., Tham, T.N., Gentry, G.A., Aubertin, A., Chinchar, V.G., 1996. Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus *Frog virus 3*. Virology 216, 431–436.

Mao, J.H., Green, D.E., Fellers, G., Chinchar, V.G., 1999. Molecular characterization of iridoviruses isolated from sympatric amphibians and fish. Virus Research 63, 45–52.

Mao, J.H., Hedrick, R.P., Chinchar, V.G., 1997. Molecular characterization, sequence analysis, and taxonomic position of newly isolated fish iridoviruses. Virology 229, 212–220.

Marschang, R.E., Braun, S., Becher, P., 2005. Isolation of a Ranavirus from a gecko (*Uroplatus fimbriatus*). Journal of Zoo and Wildlife Medicine 36, 295–300.

Marsh, I.B., Whittington, R.J., O'Rourke, B., Hyatt, A.D., Chisholm, O., 2002. Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. Molecular and Cellular Probes 16, 137–151.

Mavian, C., Lopez-Bueno, A., Balseiro, A., Casais, R., Alcami, A., Alejo, A., 2012. The Genome Sequence of the Emerging Common Midwife Toad Virus Identifies an Evolutionary Intermediate within Ranaviruses. Journal of Virology 86, 11413–11413.

May, R.M., 2004. Ecology - Ethics and amphibians. Nature 431, 403–403.

Mazzoni, R., de Mesquita, A.J., Fleury, L.F.F., Diederichsen de Brito, W.M.E., Nunes, I.A., Robert, J., Morales, H., Guedes Coelho, A.S., Barthasson, D.L.,

Galli, L., Catroxo, M.H.B., 2009. Mass mortality associated with a frog virus 3-like Ranavirus infection in farmed tadpoles *Rana catesbeiana* from Brazil. Diseases of Aquatic Organisms 86, 181–191.

Mendelson, J.R., Lips, K.R., Gagliardo, R.W., Rabb, G.B., Collins, J.P., Diffendorfer, J.E., Daszak, P., Ibanez D., R., Zippel, K.C., Lawson, D.P., Wright, K.M., Stuart, S.N., Gascon, C., da Silva, H.R., Burrowes, P.A., Joglar, R.L., La Marca, E., Loetters, S., du Preez, L.H., Weldon, C., Hyatt, A., Rodriguez-Mahecha, J.V., Hunt, S., Robertson, H., Lock, B., Raxworthy, C.J., Frost, D.R., Lacy, R.C., Alford, R.A., Campbell, J.A., Parra-Olea, G., Bolanos, F., Calvo Domingo, J.J., Halliday, T., Murphy, J.B., Wake, M.H., Coloma, L.A., Kuzmin, S.L., Price, M.S., Howell, K.M., Lau, M., Pethiyagoda, R., Boone, M., Lannoo, M.J., Blaustein, A.R., Dobson, A., Griffiths, R.A., Crump, M.L., Wake, D.B., Brodie, E.D., 2006. Biodiversity - Confronting amphibian declines and extinctions. Science 313, 48–48.

Meyer, S., 2010. Spatio-Temporal Infectious Disease Epidemiology based on Point Processes (mast). Institut für Statistik.

Meyer, S., Elias, J., Hoehle, M., 2012. A Space-Time Conditional Intensity Model for Invasive Meningococcal Disease Occurrence. Biometrics 68, 607–616.

Miller, D., Gray, M., Storfer, A., 2011. Ecopathology of Ranaviruses Infecting Amphibians. Viruses 3, 2351–2373.

Monis, P.T., Giglio, S., Saint, C.P., 2005. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Analytical Biochemistry 340, 24–34.

Moody, N., Owens, L., 1994. Experimental Demonstration of the Pathogenicity of a Frog Virus, Bohle Iridovirus, for a Fish Species, Barramundi-Lates-Calcarifer. Diseases of Aquatic Organisms 18, 95–102.

Nazir, J., Spengler, M., Marschang, R.E., 2012. Environmental persistence of amphibian and reptilian ranaviruses. Diseases of Aquatic Organisms 98, 177–184.

Normile, D., 2008. Driven to extinction. Science 319, 1606–1609.

Ostfeld, R.S., Keesing, F., 2012. Effects of Host Diversity on Infectious Disease. Annual Review of Ecology, Evolution, and Systematics, 43, 157–182.

Paperna, I., Vilenkin, M., Matos, A.P.A. de, 2001. Iridovirus infections in farm-reared tropical ornamental fish. Dis Aquat Org 48, 17–25.

Parris, K.M., McCall, S.C., McCarthy, M.A., Minteer, B.A., Steele, K., Bekessy, S., Medvecky, F., 2010. Assessing ethical trade-offs in ecological field studies. Journal of Applied Ecology 47, 227–234.

Parris, K.M., McCarthy, M.A., 2001. Identifying effects of toe clipping on anuran return rates: the importance of statistical power. Amphibia-Reptilia 22, 275–289.

Pasmans, F., Blahak, S., Martel, A., Pantchev, N., Zwart, P., 2008. Ranavirus-associated mass mortality in imported red tailed knobby newts (*Tylototriton kweichowensis*): A case report. Veterinary Journal 176, 257–259.

Picco, A.M., Brunner, J.L., Collins, J.P., 2007. Susceptibility of the endangered California tiger salamander, *Ambystoma californiense*, to *Ranavirus* infection. J. Wildl. Dis. 43, 286–290.

Picco, A.M., Collins, J.P., 2008. Amphibian Commerce as a Likely Source of Pathogen Pollution. Conservation Biology 22, 1582–1589.

Putta, S., Smith, J.J., Walker, J.A., Rondet, M., Weisrock, D.W., Monaghan, J., Samuels, A.K., Kump, K., King, D.C., Maness, N.J., Habermann, B., Tanaka, E., Bryant, S.V., Gardiner, D.M., Parichy, D.M., Voss, S.R., 2004. From biomedicine to natural history research: EST resources for ambystomatid salamanders. Bmc Genomics 5,54.

Pybus, O.G., Rambaut, A., 2009. Evolutionary analysis of the dynamics of viral infectious disease. Nature Reviews Genetics 10, 540–550.

Rachowicz, L.J., Hero, J.M., Alford, R.A., Taylor, J.W., Morgan, J. a. T., Vredenburg, V.T., Collins, J.P., Briggs, C.J., 2005. The novel and endemic pathogen hypotheses: Competing explanations for the origin of emerging infectious diseases of wildlife. Conservation Biology 19, 1441–1448.

Rachowicz, L.J., Knapp, R.A., Morgan, J.A.T., Stice, M.J., Vredenburg, V.T., Parker, J.M., Briggs, C.J., 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. Ecology 87, 1671–1683.

Raffel, T.R., Rohr, J.R., Kiesecker, J.M., Hudson, P.J., 2006. Negative effects of changing temperature on amphibian immunity under field conditions. Functional Ecology 20, 819–828.

Raffel, T.R., Romansic, J.M., Halstead, N.T., McMahon, T.A., Venesky, M.D., Rohr, J.R., 2013. Disease and thermal acclimation in a more variable and unpredictable climate. Nature Climate Change 3, 146–151.

Rambaut, A., & Drummond, A. (2009). FigTree v1. 3.1.

Reading, C.J., 1998. The effect of winter temperatures on the timing of breeding activity in the common toad Bufo bufo. Oecologia 117, 469–475.

Reading, C.J., 2007. Linking global warming to amphibian declines through its effects on female body condition and survivorship. Oecologia 151, 125–131.

Reed, L.J., Muench, H., 1938. A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Epidemiol. 27, 493–497.

Ridenhour, B.J., Storfer, A.T., 2008. Geographically variable selection in *Ambystoma tigrinum virus* (*Iridoviridae*) throughout the western USA. J. Evol. Biol. 21, 1151–1159.

Robert, J., Abramowitz, L., Gantress, J., Morales, H.D., 2007. *Xenopus laevis*: A possible vector of ranavirus infection? Journal of Wildlife Diseases 43, 645–652.

Rojas, S., Richards, K., Jancovich, J.K., Davidson, E.W., 2005. Influence of temperature on Ranavirus infection in larval salamanders *Ambystoma tigrinum*. Diseases of Aquatic Organisms 63, 95–100.

Rothenburg, S., Chinchar, V.G., Dever, T.E., 2011. Characterization of a ranavirus inhibitor of the antiviral protein kinase PKR. BMC Microbiology 11, 56.

Rupprecht, C., Smith, J., Fekadu, M., Childs, J., 1995. The Ascension of Wildlife Rabies - a Cause for Public-Health Concern or Intervention. Emerg. Infect. Dis. 1, 107–114.

Schloegel, L.M., Daszak, P., Cunningham, A.A., Speare, R., Hill, B., 2010. Two amphibian diseases, chytridiomycosis and ranaviral disease, are now globally notifiable to the World Organization for Animal Health (OIE): an assessment. Diseases of Aquatic Organisms 92, 101–108.

Schloegel, L.M., Picco, A.M., Kilpatrick, A.M., Davies, A.J., Hyatt, A.D., Daszak, P., 2009. Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*). Biological Conservation 142, 1420–1426.

Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., Zhao, S., Wangen, C., Ferreira, C.M., Hipolito, M., Davies, A.J., Cuomo, C.A., Daszak, P., James, T.Y., 2012. Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Molecular Ecology 21, 5162–5177.

Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors – occurrence, properties and removal. J. Appl. Microbiol. 113, 1014–1026.

Shackelton, L.A., Holmes, E.C., 2004. The evolution of large DNA viruses: combining genomic information of viruses and their hosts. Trends in Microbiology 12, 458–465.

Sharifian-Fard, M., Pasmans, F., Adriaensen, C., Devisscher, S., Adriaens, T., Louette, G., Martel, A., 2011. Ranavirosis in Invasive Bullfrogs, Belgium. Emerging Infectious Diseases 17, 2371–2372.

Sharp, P.M., Bailes, E., Chaudhuri, R.R., Rodenburg, C.M., Santiago, M.O., Hahn, B.H., 2001. The origins of acquired immune deficiency syndrome viruses: where and when? Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci. 356, 867–876.

Speare, R., Smith, J., 1992. An Iridovirus-Like Agent Isolated from the Ornate Burrowing Frog *Limnodynastes ornatus* in Northern Australia. Diseases of Aquatic Organisms 14, 51–57.

St-Ainour, V., Lesbarreres, D., 2007. Genetic evidence of Ranavirus in toe clips: an alternative to lethal sampling methods. Conservation Genetics 8, 1247–1250.

St-Amour, V., Wong, W.M., Garner, T.W.J., Lesbarreres, D., 2008. Anthropogenic influence on prevalence of 2 amphibian pathogens. Emerging Infectious Diseases 14, 1175–1176.

Stöhr, A.C., Hoffmann, A., Papp, T., Robert, N., Pruvost, N.B.M., Reyer, H.-U., Marschang, R.E., 2013. Long-term study of an infection with ranaviruses in a group of edible frogs (*Pelophylax kl. esculentus*) and partial characterization of two viruses based on four genomic regions. The Veterinary Journal 197, 238–244.

Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S.L., Fischman, D.L., Waller, R.W., 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306, 1783–1786.

Tan, W.G.H., Barkman, T.J., Chinchar, V.G., Essani, K., 2004. Comparative genomic analyses of *Frog virus 3*, type species of the genus Ranavirus (family *Iridoviridae*). Virology 323, 70–84.

Taylor, L.H., Latham, S.M., Woolhouse, M.E.J., 2001. Risk factors for human disease emergence. Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci. 356, 983–989.

Teacher, A.G.F., Cunningham, A.A., Garner, T.W.J., 2010. Assessing the long-term impact of Ranavirus infection in wild common frog populations. Animal Conservation 13, 514–522.

Teacher, A.G.F., Garner, T.W.J., Nichols, R.A., 2009a. Evidence for Directional Selection at a Novel Major Histocompatibility Class I Marker in Wild Common Frogs (*Rana temporaria*) Exposed to a Viral Pathogen (*Ranavirus*). PLoS One 4(2), e4616.

Teacher, A.G.F., Garner, T.W.J., Nichols, R.A., 2009b. Population genetic patterns suggest a behavioural change in wild common frogs (*Rana temporaria*) following disease outbreaks (*Ranavirus*). Molecular Ecology 18, 3163–3172.

Tidona, C.A., Schnitzler, P., Kehm, R., Darai, G., 1998. Is the major capsid protein of iridoviruses a suitable target for the study of viral evolution? Virus Genes 16, 59–66.

Timms, R., Colegrave, N., Chan, B.H.K., Read, A.F., 2001. The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. Parasitology 123, 1–11.

Tsai, C.T., Ting, J.W., Wu, M.H., Wu, M.F., Guo, I.C., Chang, C.Y., 2005. Complete genome sequence of the *Grouper iridovirus* and comparison of genomic organization with those of other iridoviruses. Journal of Virology 79, 2010–2023.

Une, Y., Sakuma, A., Matsueda, H., Nakai, K., Murakami, M., 2009. Ranavirus Outbreak in North American Bullfrogs (*Rana catesbeiana*), Japan, 2008. Emerging Infectious Diseases 15, 1146–1147.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3-new capabilities and interfaces. Nucleic Acids Research 40(15), e115.

VanCompernolle, S.E., Taylor, R.J., Oswald-Richter, K., Jiang, J.Y., Youree, B.E., Bowie, J.H., Tyler, M.J., Conlon, J.M., Wade, D., Aiken, C., Dermody, T.S.,

KewalRamani, V.N., Rollins-Smith, L.A., Unutmaz, D., 2005. Antimicrobial peptides from amphibian skin potently inhibit human immunodeficiency virus infection and transfer of virus from dendritic cells to T cells. J. Virol. 79, 11598–11606.

Van Strien, A., Pannekoek, J., Hagemeijer, W., Verstrael, T., 2000. A Loglinear Poisson Regression Method To Analyse Bird Monitoring Data. Bird Census News 13, 33–39.

Vesely, T., Cinkova, K., Reschova, S., Gobbo, F., Ariel, E., Vicenova, M., Pokorova, D., Kulich, P., Bovo, G., 2011. Investigation of ornamental fish entering the EU for the presence of ranaviruses. Journal of Fish Diseases 34, 159–166.

Wake, D., 1991. Declining Amphibian Populations. Science 253, 860–860.

Walker, S.F., Bosch, J., Gomez, V., Garner, T.W.J., Cunningham, A.A., Schmeller, D.S., Ninyerola, M., Henk, D.A., Ginestet, C.E., Arthur, C.-P., Fisher, M.C., 2010. Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecology Letters 13, 372–382.

Walker, S.F., Bosch, J., James, T.Y., Litvintseva, A.P., Oliver Valls, J.A., Pina, S., Garcia, G., Abadie Rosa, G., Cunningham, A.A., Hole, S., Griffiths, R., Fisher, M.C., 2008. Invasive pathogens threaten species recovery programs. Curr. Biol. 18, R853–R854.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M., Barton, G.J., 2009. Jalview Version 2-a multiple sequence alignment editor and analysis workbench. Bioinformatics 25, 1189–1191.

Weissenberg, R., 1965. Fifty years of research on the lymphocystis virus disease of fishes (1914-1964). Annals of the New York Academy of Sciences 126, 362–374.

Whiles, M.R., Lips, K.R., Pringle, C.M., Kilham, S.S., Bixby, R.J., Brenes, R., Connelly, S., Colon-Gaud, J.C., Hunte-Brown, M., Huryn, A.D., Montgomery, C., Peterson, S., 2006. The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. Front. Ecol. Environ. 4, 27–34.

Whittington, R.J., Chong, R., 2007. Global trade in ornamental fish from an Australian perspective: The case for revised import risk analysis and management strategies. Preventive Veterinary Medicine 81, 92–116.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63, 3741–3751.

Wood, J.L.N., Leach, M., Waldman, L., MacGregor, H., Fooks, A.R., Jones, K.E., Restif, O., Dechmann, D., Hayman, D.T.S., Baker, K.S., Peel, A.J., Kamins, A.O., Fahr, J., Ntiamoa-Baidu, Y., Suu-Ire, R., Breiman, R.F., Epstein, J.H., Field, H.E., Cunningham, A.A., 2012. A framework for the study of zoonotic disease emergence and its drivers: spillover of bat pathogens as a case study. Philos. Trans. R. Soc. B-Biol. Sci. 367, 2881–2892.

Woolhouse, M.E.J., Taylor, L.H., Haydon, D.T., 2001. Population biology of multihost pathogens. Science 292, 1109–1112.

Xu, K., Zhu, D.-Z., Wei, Y., Schloegel, L.M., Chen, X.-F., Wang, X.-L., 2010. Broad Distribution of *Ranavirus* in Free-Ranging *Rana dybowskii* in Heilongjiang, China. Ecohealth 7, 18–23.

Zhang, Q.Y., Li, Z.Q., Gui, J.F., 1999. Studies on morphogenesis and cellular interactions of *Rana grylio virus* in an infected fish cell line. Aquaculture 175, 185–197.

Zhang, Q.Y., Xiao, F., Li, Z.Q., Gui, J.F., Mao, J.H., Chinchar, V.G., 2001. Characterization of an iridovirus from the cultured pig frog Rana grylio with lethal syndrome. Diseases of Aquatic Organisms 48, 27–36.

Zhou, Z.Y., Geng, Y., Liu, X.X., Ren, S.Y., Zhou, Y., Wang, K.Y., Huang, X.L., Chen, D.F., Peng, X., Lai, W.M., 2013. Characterization of a ranavirus isolated from the Chinese giant salamander (Andrias davidianus, Blanchard, 1871) in China. Aquaculture 384, 66–73.